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Contents

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Contents

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REVIEW

Effect of aging on stem cells

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Abstract

Pluripotent stem cells have the remarkable self-renewal ability and are capable of differentiating into multiple diverse cells. There is increasing evidence that the aging process can have adverse effects on stem cells. As stem cells age, their renewal ability deteriorates and their ability to differentiate into the various cell types is altered. Accordingly, it is suggested aging-induced deterioration of stem cell functions may play a key role in the pathophysiology of the various aging-associated disorders. Understanding the role of the aging process in deterioration of stem cell function is crucial, not only in understanding the pathophysiology of aging-associated disorders, but also in future development of novel effective stem cell-based therapies to treat aging-associated diseases. This review article first focuses on the basis of the various aging disease-related stem cell dysfunction. It then addresses the several concepts on the potential mechanism that causes aging-related stem cell dysfunction. It also briefly discusses the current potential therapies under development for aging-associated stem cell defects.

Key words: Aging; Biological aging; Cellular aging; Adult stem cells; Premature aging; Mesenchymal stem cell; Stem cell renewal; Tissue regeneration

Core tip: Stem cells have the remarkable self-renewal capability and the amazing ability to differentiate into all cell types. It is generally believe that stem cells are the main source that provides cells to repair and re-generate damaged tissues and organs. However, there is now compelling evidence that the aging process has a deleterious effect on stem cells, and that the aging effects on stem cells may have play essential roles in the pathophysiology of the various aging-associated diseases. This review discusses briefly the relationship of aging-

associated stem cell dysfunction and the various aging-associated ailments, and several proposed concepts on the molecular mechanism of aging-related stem cell dysfunction.

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INTRODUCTION

Aging is an unavoidable physiological consequence of the living animals. Mammalian aging is mediated by the complex cellular and organismal processes, driven by diverse acquired and genetic factors[1]. Aging is among the greatest known risk factors for most human diseases[2-5], and of roughly 150000 people who die each day across the globe, about two thirds die from age-related causes[6].

In modern era, one of the emerging fields in treating human diseases is the "stem cells" research, as stem cells have the remarkable potential for use to treat a wide range of diseases. Accordingly, stem cells research has become a focal point of biomedical research since 1998, when Dr. James Alexander Thomson made the scientific breakthrough of successful generation of several embryonic stem cell lines from human blastocysts[7,8]. Stem cells are undifferentiated pluripotent cells that can give rise to all tissue types and serve as a sort of internal repair system[9]. Until the recent advance in development of induced pluripotent stem cells (iPSCs), scientists primarily worked with two kinds of pluripotent stem cells from animals and humans: Embryonic stem cells, which are isolated from the inner cell mass of blastocysts, and non-embryonic "somatic" or "adult" stem cells, which are found in various tissues[10]. Because of potential ethical issues, "adult" stem cells have become the primary target.

Although stem cell science promises to offer revolutionary new ways of treating diseases, it is identified that aging affect the ability of stem (and progenitor) cells to function properly, which ultimately can lead to cell death (apoptosis), senescence (loss of a cell's power of division and growth), or loss of regenerative potential[11,12]. Aging may also shift gene functions, as reported for some genes such as, p53 and mammalian target of rapamycin (mTOR), which are beneficial in early life, but becomes detrimental later in life[13-15]. In this regard, a novel theory, namely "stem cell theory of aging", has been formulated, and it assumes that inability of various types of pluripotent stem cells to continue to replenish the tissues of an organism with sufficient numbers of appropriate functional differentiated cell types capable of maintaining that tissue's (or organ's) original function is in large part responsible for the aging process[1]. In addition, aging also compromises the therapeutic potentials of stem cells, including cells isolated from aged individuals or cells that had been cultured for many passages *in vitro*. Nevertheless, in either case, understanding the molecular mechanism involved in aging and deterioration of stem cell function is crucial in developing effective new therapies for aging- as well as stem cell malfunction-related diseases. In fact, given the importance of the aging-associated diseases, scientists have developed a keen interest in understanding the aging process as well as attempting to define the role of dysfunctional stem cells in the aging process.

In this review, we will first focus on the various aging disease-related stem cell dysfunction and then address the several concepts on potential mechanisms that cause aging-related stem cell dysfunction. We will also discuss current strategies for reversing age-related stem cell dysfunction. Finally, we will discuss up-to-date therapies for aging-associated stem cell defects, available-drugs, growth factors, *etc*.

DISEASES OF AGING FROM OLD STEM CELLS

Adult stem cells, also known as somatic stem cells, are found throughout the body in every tissues and organs after development and function as self-renewing cell pools to replenish dying cells and regenerate damaged tissues throughout life[16]. However, adult stem cells appear to age with the person. As stem cells age, their functional ability also deteriorates[12,17]. Specifically, this regenerative power appears to decline with age, as injuries in older individuals heal more slowly than in childhood. For example, healing of a fractured bone takes much longer time in elderly than in young individuals[18-21]. There is a substantial amount of evidence showing that deterioration of adult stem cells in the adult phase can become an important player in the initiation of several diseases in aging[22,23]. The following is some of the examples of aging-associated effects on stem cells.

Neural stem cells (NSCs) are multipotent and self-renewing cells and located primarily in the neural tissues. In response to a complex combination of signaling pathways, NSCs differentiate into various specific cell types locally in the central nervous system (CNS), like neurons, astrocytes, and oligodendrocytes[24]. NSCs in humans maintain brain homeostasis and it continuously replenishes new neurons, which are important for cognitive functions[25,26]. However, there is now strong evidence for the aging-associated cognitive deficits, such as olfactory dysfunction, spatial memory deficits, and neurodegenerative disorders, which are caused by deterioration of NSC proliferation and differentiation and enhanced NSC senescence as a consequence of aging[27,28].

Mesenchymal stem cells (MSCs) are multipotent stromal cells that can differentiate into cells of mesenchyme tissues, including osteoblasts (bone cells)[29], chondrocytes (cartilage

cells)[30], myocytes (muscle cells)[31] and adipocytes (fat cells)[32]. MSCs were first isolated from the bone marrow of guinea pigs in 1970's and after that it was isolated from almost every organ in mice including fat, liver, spleen, pancreas, kidney, lung, muscle, and brain[32]. Human MSCs have also been isolated from umbilical cord tissue and cord blood, placenta, bone and joints[33]. However, the major sources of MSCs are the bone marrow-derived MSCs (BM-MSCs) and the adipose tissue-derived MSCs (A-MSCs); and they are currently the most studied MSCs[32,34]. Aging also affects MSCs in humans and in animal models as indicated by the decrease in the bone marrow MSC pool and also shifts their lineage differentiation from one that usually favors osteoblastic differentiation to one that prefers adipogenic differentiation[35], which is largely responsible for the gradual and aging-associated shift of hematopoietic (red) marrows to fatty (yellow) marrows, and which also contributes significantly to the etiology of senile osteoporosis. It is also evident that with increasing donor age, MSCs from both bone marrow and adipose tissues have been shown to have reduced capacity to handle oxidative stress[36-38]. During the aging process, oxidative stress leads to hyperactivity of pro-growth pathways, such as insulin/IGF-1 and mTOR pathways, and the subsequent accumulation of toxic aggregates and cellular debris ultimately lead to apoptosis, necrosis, or autophagy[39]. In addition, in some non-skeletal tissues, particularly the hematopoietic system, MSCs is a key niche component for hematopoietic cells. Aging of MSCs has been shown to be detrimental with respect to this important function[35].

Adult skeletal muscle stem cells (satellite cells) have a remarkable capacity to regenerate[40,41]. Similarly, their regeneration capacity declines with aging, although it is not clear whether this is due to extrinsic changes in the environment and/or to cell-intrinsic mechanisms associated to aging. This impaired regenerative capacity of skeletal muscle during aging is due to accumulation of the altered progeny, which leads to progressive deterioration of tissue structure and function, manifesting after injury or in response to the depletion of memory B cells and naive T cells in the hematopoietic system in the elderly[41-44].

Hematopoietic stem cells (HSCs) are the blood-forming stem cells through the process of hematopoiesis[45]. They are located in the red bone marrow within marrow cavity of most bones. HSCs also produce immune cells of the body. Since blood cells are responsible for constant maintenance and immune protection of every cell type of the body, the constant production of billions of new blood cells each day by HSCs is very important for mammal life. HSC-derived monocytes can give rise to osteoclasts, macrophage and granulocyte. Osteoclasts are giant cells with numerous nuclei that work in synergy with osteoblasts through complicated bone coupling mechanisms to maintain bone homeostasis[35,46]. All these activities of HSCs are carefully modulated by a complex interplay between cell-intrinsic mechanisms and cell-extrinsic factors produced by the microenvironment; and aging altered this fine-tuned regulatory network, leading to aberrant HSC cell cycle regulation, degraded HSC function, and hematological malignancy[47].

MECHANISM FOR FUNCTIONAL DETERIORATION OF STEM CELL IN AGING

There are several potential mechanisms that are believed to contribute to the aging-associated stem cell dysfunction; and they probably are in part responsible for many aging-associated diseases. Figure 1 proposes some of the contributing factors/mechanisms that could be responsible for the aging-induced deterioration of stem cell functions and aging-associated diseases. This section summarizes some of these contributing factors/mechanisms and their potential roles in the aging effect on stem cells.

Microenvironment

Aging is characterized by common environmental conditions, such as hormonal, immunologic, and metabolic disorders[48-50] and these are considered as the critical microenvironmental factors affecting stem cell functions. Changes in these microenvironmental factors in response to aging are believed to be responsible for the changes in stem cell function with aging[51]. It has been shown that potentially underlying aging-related tissue degeneration, such as osteoporosis, could be due to impaired MSCs by surrounding micro-environmental pathologic factors[52,53]. It has also been shown that in mammals, metabolic alterations of hyperglycemia and hyperinsulinemia are important pathologic factors in aging and in MSC dysfunction[54]. However, the molecular mechanism in mediating stem cells dysfunction by microenvironmental signals is not yet fully understood.

Cells produce soluble (endocrine or paracrine) factors necessary for information exchange among cells of distant tissues and/or within the same organ[51]. Aging cells can influence an organ or tissue by secreting soluble endocrine or paracrine factors. Accordingly, aging of the endocrine glands has known to result in hormonal disturbances[50,55], which ultimately affects normal function and or differentiation of the stem cells. In humans, sex hormones, especially estrogen, are the most prominent endocrine factors that change with aging, and sex hormones discordance often leads to several significant diseases. Estrogen insufficiency also induces the biased differentiation of MSCs to adipocytes over osteoblasts[50,56,57]. Aging-related elevation in circulating levels of proinflammatory cytokines, such as interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α), can also cause differentiation disorders of MSCs[58,59].

DNA damage and telomere shortening

In mammals, spontaneous and extrinsic mutational

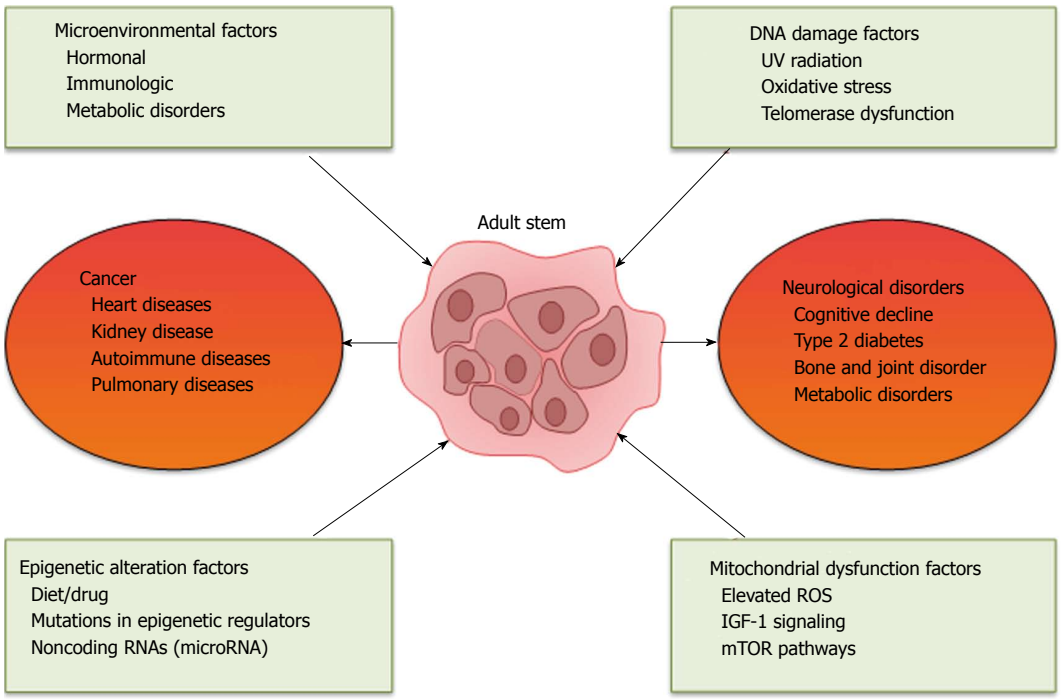

Figure 1 A proposed mechanism for the aging-induced deterioration of stem cell functions and aging-associated diseases. ROS: Reactive oxygen species.

events occur on DNA on daily basis. While most of the damaged DNAs are repaired by normal DNA repair mechanism, some of the mutated DNAs appear to escape from the repair mechanism and accumulate over time. Accordingly, there would be a significant accumulation of mutated or damaged DNAs in aging cells compared to young cells. The accumulation of damaged DNA may in part be responsible for the various cellular events of the aging process. In fact, this "mutational theory" is one of the earliest theories of the aging process[15]. DNA damage can be caused by environmental factors, like UV irradiation, and also can be the consequence of the cell's own metabolic processes [*e.g.*, generating reactive oxygen species (ROS)] that tend to accumulate with time[60]. DNA damage impaired stem-cell function in aging, which has been documented by the study that HSCs derived from aged mice harbored significant alterations in their DNA repair response[1,17]. DNA-repair proteins, such as FANCD1[61], MSH2[62] or ERCC1[63], are found to be deficient in adult mice with significant functional defects of HSCs and the dysfunction of MSCs in aging led to leukemia and aging-associated remodeling[64]. In addition, measures of DNA damage in HSCs, such as histone H2AX phosphorylation and comet tails, were also found to be increased with advancing age[65,66]. In satellite cells, H2AX phosphorylation was also accumulated with increasing age[67].

Premature aging can be resulted from defects in the DNA repair and telomerase pathway components in humans and mice[68]. In aging diseases, there has been significant interest in the telomere shortening that is now being used as a hallmark of aging, to which even stem cells are not immune[1,17]. A telomere is a region of repetitive nucleotide sequences at each end of a chromosome. It protects genome from nucleolytic degradation, unnecessary recombination, repair, or fusion with neighboring chromosomes[69]. Although stem cells express telomerase, the telomeres of HSCs, MSCs, NSCs, HFSCs and GSCs do shorten with age[70-72]. When telomeres become critically short, the cell becomes senescent, it ceases to divide and may undergo apoptosis. In fact, many aging-associated diseases, like the increased cancer risk[73,74], coronary heart disease[75-77], heart failure[78], diabetes[79], and osteoporosis[80], are caused by accelerated telomere shortening. Despite considerable evidence that telomere shortening causes reduction in life span, the telomere shortening concept of aging is still somewhat controversial, since laboratory mice lacking telomerase RNA component (TERC) showed no obvious abnormal phenotypes even after five generations[81,82].

Mitochondrial dysfunction

Mitochondria are ubiquitous intracellular organelles in mammals and are the main source of cellular adenosine triphosphate (ATP) that plays a central role in a variety of cellular processes. As mitochondria produce about 90% of cellular energy, the aging-related ROS generation, disruption in Ca^{2+} homeostasis, and increased cell apoptosis are three causes of mitochondria dysfunction that directly affects aging-related diseases[83]. In fact, there have been many studies suggesting a direct relationship between mitochondrial dysfunction and human stem cell aging[84-87]. Accordingly, in several cell systems, mitochondrial dysfunction has been shown to lead to respiratory chain dysfunction, which may be the result of the accumulation of mutations in mitochondrial DNA

(mtDNA)[88]. The elevated ROS in aging is mainly due to mtDNA mutation, as mitochondria is the primary cellular sources of ROS[89]. In addition, it has been confirmed that mitochondrial aging interact with other cellular pathways of aging, such as the IGF-1 signaling and the mTOR pathways, which presumed to play a major role in aging[90,91].

Epigenetic alteration

Epigenetics refer to changes in gene expression, which are heritable through modifications without affecting the DNA sequence. It has also been defined more broadly as the dynamic regulation of gene expression by sequence-independent mechanisms, including but not limited to changes in DNA methylation and histone modifications[92-94]. Epigenetic marks in stem cells are transmitted heritably to their daughter cells, priming lineage-specific loci for modification in downstream progenies[95]. Stem cell fates are regulated by epigenetic modifications of DNA that establish the memory of active and silent gene states[96,97]. Aberrant epigenetic regulation affects the organismal aging[98], age-associated dysfunction of stem cells, and predisposition to hematological cancers development[99]. For instance, DNA methylation specific to regions of the genome that are important for lineage-specific gene expression increased in aging HSCs[100] and the perturbations of their histone modifications (H3K4me3) may impair its self-renewal genes[101]. It has also been reported that mutations in epigenetic regulators, such as DNMT3a, TET2, and ASXL1, are frequently found in myeloid neoplasia[102]. Since most of the chromatin changes are intrinsically reversible, epigenetic alterations are therefore considered good therapeutic targets for molecular effectors and thereby are potential therapies for certain distinct pathologies[103,104]. Therefore, there has been immense interest in understanding these genome-scale regulatory mechanisms that lead to impaired gene expression, and that contribute to the decline of stem cell and tissue function with age.

MicroRNAs (miRNAs) are another key class of epigenetic mediators of stem cell dysfunction. They are a class of small noncoding RNAs composed of 18- to 25-bp nucleotides[105] that functions in RNA silencing and post-transcriptional regulation of gene expression[106-109]. It plays an important role in regulating stem cell self-renewal and differentiation by repressing the translation of selected mRNAs in stem cells and differentiating daughter cells[110]. In fact, non-coding RNA-mediated regulatory events as a part of the epigenetic mechanism to modulate mRNA degradation and/or protein translation that play important role in development and disease state[111]. MiRNAs, such as miR-17, regulates osteoblast differentiation of MSCs[112-114]. MiR-290–295 cluster seems to promote embryonic stem cell differentiation, self-renewal, and maintenance of pluripotency[110,115]. Moreover, recent findings show the involvement of miRNAs in senescence manipulation. These findings have led to the suggested use of these miRNAs as clinical biomarkers of stem cell senescence and their potentiality[116].

THERAPEUTIC APPROACHES FOR THE TREATMENT OF AGING-INDUCED STEM CELL DYSFUNCTION

In recent years with increasing understanding of stem cell behavior in different niche of the body offers promise for the development of potential therapeutic approaches to treat aging-associated dysregulation of adult stem cells and aging-related diseases. Some of the potential therapeutic approaches for the treatment of age-related stem cell dysfunction are discussed below.

Parabiosis

The concept of parabiosis is not new; however, in the past decade its role in reversing the effects of aging and enhancing rejuvenation has gathered substantial momentum. Recent findings suggest that aging-related cellular dysfunctions can be repaired successfully by modulating the molecular architecture of the tissue environment rather than inducing cell intrinsic changes alone[117]. Therefore, the effects of aging in an old individual can be modulated or reversed by the circulatory or systemic factors derived from the young blood through anatomical joining, parabiosis[40]. The fascinating results of parabiosis have been reported to rejenuvate brain[118], muscles[67], and liver tissues in the aged animals[119]. In skeletal muscle regeneration, serum derived from young mice activated the Notch signaling pathway and regulated the satellite cells proliferation of old mice *in vitro*[119]. In aged mice, through the parabiosis approach, systemic factors from young mice successfully reversed inefficient CNS remyelination, a regenerative process of CNS that produces new myelin sheaths from adult stem cells[118]. Despite the promising outcomes in animal models, there is persistence of contradiction in functions of factors identified in prominent parabiosis studies, rendering the concept highly controversial for use in humans. For instance, growth differentiation factor 11 (GDF-11) has been reported to show both positive[67] and negative corelations[120] with stem cell aging.

Retrotansposons

Retrotansposons are mobile DNA elements that can induce genetic instability and have been reported to be a cause of cellular dysfunction during aging[121]. The long interspaced nuclear elements (L1) are 6-kb long retrotransposons that code for RNA binding protein and endonuclease protein. There are 500000 copies of L1 elements in the human genome, and approximately 100 of such active elements replicated to induce genomic instabilities and to increase the risk of DNA damage. Elevated activity of L1 has been reported in aging-related pathological conditions[122]. The link between SIRT-6 (an important marker of longevity) and L1 offered more direct evidence for the role of L1 in aging-related genomic complications. SIRT6 are

known to repress the activity of L1 retrotransposons[123]. DNA damage-induced mobilization of SIRT6 to the site of repair and subsequent repression of L1 have been contemplated in the development of therapeutics for age-related neurological pathologies, such as dementia and cancer[124]. Suppression of L1 activity by overexpression of SIRT6 in senescence cells delayed the onset of L1-induced pathological conditions. High caloric diet activated the SIRT1 activity and has been reported to protect the animal from premature aging in Cockayne syndrome[125], whereas in the case of the mouse Alzheimer's disease model the caloric restriction slowed down the disease progression[126]. Other than modulation of SIRT6 expression, inhibition of reverse transcriptase (a critical enzyme for the L1 replication) is another way to attenuate L1 activity[127]. Several small non-coding RNAs, such as pi-RNAs, si-RNAs and L1 specific small RNAs, have also been reported to regulate the silencing of retrotransposons element activity in mouse germ cells and in aging human somatic cells[127].

Cellular reprogramming towards iPSCs

iPSCs are a type of pluripotent stem cell that can be generated directly from adult cells and the recent advances in this area have opened up many gateways for the research in cell-based therapeutics[128]. Cellular reprogramming of aged somatic cells towards iPSC enables the editing and resetting of the cellular clock by removing the characteristic feature of aging. The ability to derive iPSCs from aging-related pathological cells have enabled investigators to develop recombination-based therapeutic approaches to edit genetic defects responsible of premature and accelerated aging. The reprogramming of aged somatic cells to target stem can be used as an alternative source to get cells for transplantation and for genetic editing. Recent studies show encouraging effects of reprogramming in rejuvenation of senescent cells, as evident by elongated telomeres and reduced oxidative stress[129]. Human iPSC-based models for aging-related degenerative diseases have been tested to understand the disease dynamics in Parkinson's disease, Alzheimer's disease and in progeroid laminopathies[130]. Valuable information from these studies has resulted in the first clinical trial for progeroid patients[131]. In a mouse model of skeletal defect, human iPSC designed to express PAX7 were able to be differentiated into muscle progenitor cells that engrafted and repaired the defective dystrophin-positive myofibers formation. In case of Hutchinson-Gilford progeria syndrome (HGPS), reprogramming of HGPS fibroblasts by transduction with vectors expressing Oct4, Sox2, Klf4 and c-Myc has been reported to revert aging-associated markers, such as Lamin, to a "young" state[132].

Telomere lengthening

As discussed above, the telomere length is inversely linked to the chronical age, and thus it is believed that increasing the length of telomere may increase life span. Many advanced approaches are being developed to efficiently increase the telomere length and to protect cells from chromosome shortening. In *in vitro* cultured human cells, the delivery of RNA coding for telomere-extending protein has been reported to increase the cell proliferation rate[133]. In telomere-deficient mice, genetic editing to reactivate telomerase activity has been reported to reverse the aging symptoms[134]. Telomerase activation drugs and telomerase gene therapy are also alternative approaches that aim to increase the telomere length to protect the cells from premature aging[135,136].

CONCLUSION

From the various advances in stem cell research, it is clear that we grow old partly because our stem cells grow old with us. The functions of aged stem cells become impaired as the result of cell-intrinsic pathways and surrounding environmental changes. With the sharp rise in the aging-associated diseases, the need for effective regenerative medicine strategies for the aged is more important than ever. Fortunately, rapid advances in stem cell and regenerative medicine technologies continue to provide us with a better understanding of the diseases that allows us to develop more effective therapies and diagnostic technologies to better treat aged patients. However, there is a big ethical concern regarding the use of human embryos to procure embryonic stem cells and many countries already currently restrict experiments on embryos to the first 14 d. Additionally, the International Society for Stem Cell Research has issued guidelines advising researchers across the globe to stick with this 14-d window. Nevertheless, it seems that the human stem cell research in the next decade will likely bring enormous progress in the aging-associated disease therapies but may also reach a step closer to the edge of ethical concern of creation of "Frankenstein".

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REVIEW

Odd couple: The unexpected partnership of glucocorticoid hormones and cysteinyl-leukotrienes in the extrinsic regulation of murine bone-marrow eosinopoiesis

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Abstract

Granulopoiesis in murine bone-marrow is regulated by both intrinsic and extrinsic factors (including hormones, drugs, inflammatory mediators and cytokines). Eosinophils, a minor subpopulation of circulating leukocytes, which remains better understood in its contributions to tissue injury in allergic disease than in its presumably beneficial actions in host defense, provide a striking example of joint regulation of granulopoiesis within murine bone-marrow by all of these classes of extrinsic factors. We first described the upregulation of eosinopoiesis in bone-marrow of allergen-sensitized mice following airway allergen challenge. Over the last decade, we were able to show a critical role for endogenous glucocorticoid hormones and cytokines in mediating this phenomenon through modification of cytokine effects, thereby supporting a positive association between stress hormones and allergic reactions. We have further shown that cysteinyl-leukotrienes (CysLT), a major proinflammatory class of lipid mediators, generated through the 5-lipoxygenase pathway, upregulate bone-marrow eosinopoiesis *in vivo* and *in vitro*. CysLT mediate the positive effects of drugs (indomethacin and aspirin) and of proallergic cytokines (eotaxin/CCL11 and interleukin-13) on *in vitro* eosinopoiesis. While these actions of endogenous GC and CysLT might seem unrelated and even antagonistic, we demonstrated a critical partnership of these mediators *in vivo*, shedding light on mechanisms linking stress to allergy: GC are required for CysLT-mediated upregulation of bone-marrow eosinopoiesis *in vivo*, but also attenuate subsequent *ex vivo* responses to CysLT. GC and CysLT therefore work together to induce eosinophilia, but through subtle regulatory mechanisms also limit the magnitude of subsequent bone-marrow responses to allergen.

Key words: Bone marrow; Leukotriene; Eosinophil; Stress; Glucocorticoid

Core tip: The bone-marrow is exquisitely sensitive to regulation by systemic events, which selectively increase

production of different blood cell types to meet transient increases in demand following injury. An association between stress and allergy has long been known, but its mechanisms remain incompletely understood. The exploration of underlying mechanisms in a variety of murine models yielded evidence of separate but interrelated roles for adrenal glucocorticoid hormones and cysteinyl-leukotrienes in coupling systemic events to bone-marrow responses *in vivo*. We here discuss how these unlikely partners work together to promote eosinophilia but through subtle mechanisms also limit its magnitude.

Xavier-Elsas P, Masid-de-Brito D, Vieira BM, Gaspar-Elsas MIC. Odd couple: The unexpected partnership of glucocorticoid hormones and cysteinyl-leukotrienes in the extrinsic regulation of murine bone-marrow eosinopoiesis. *World J Exp Med* 2017; 7(1): 11-24 Available from: URL: http://www.wjgnet.com/2220-315X/full/v7/i1/11.htm DOI: http://dx.doi.org/10.5493/wjem.v7.i1.11

BONE-MARROW REGULATION BY INTRINSIC AND EXTRINSIC FACTORS

In both humans and mice, the lifelong production of blood cells (definitive hemopoiesis[1,2]; takes place in the bone-marrow of long bones, and encompasses the production, from a pool of hemopoietic stem cells (HSC), of both lymphoid (B cells, Natural Killer cells) and myeloid (erythroid, megakaryocytic, mononuclear phagocyte and granulocyte) lineages, through a series of increasingly committed (specialized, or developmentally restricted) stages, recognizable as morphologically, cytochemically and/or immunophenotypically distinct cell types[1,3-5]. Mature cells may then be exported to the circulation and remain there, until they are removed during their passage through the spleen (a typical fate for erythrocytes and platelets[6]) or emigrate to the tissues, ultimately undergoing apoptosis and clearance by resident phagocytes[7]. Emigration occurs either when inflammation follows injury (thereby allowing neutrophil granulocytes to exert short-term protective functions, in the absence or presence of infection[8]; or when chemoattractants selectively expressed in some sites attract leukocytes from a particular lineage (for instance, in the case of eosinophil granulocytes, enabling them to enter the mucosa of the digestive, respiratory and reproductive tracts to become long-term resident effector and regulatory cells[9].

Usually, peripheral clearance of senescent or apoptotic cells of bone-marrow origin is coupled to replenishment by a variety of mechanisms[10]. This is no small achievement, because specialized hemopoietic cell lineages, though ultimately derived from the same pool of pluripotent, self-renewing stem cells, differ largely in their numbers, requirements and properties[1-5]; accordingly, no single mechanism can account for the maintenance of their proportions across different compartments, nor for their lineage-selective increases or decreases often observed in immune reactions and disease[4,5,11,12].

Multiple factors intrinsic to the adult bone-marrow contribute to the maintenance of a steady output of these different cell types in very disparate proportions and rates. A major intrinsic factor is the differential expansion of hemopoietic lineages, driven by intense proliferation of lineage-committed progenitors (quantifiable *in vitro* as colony-forming units, or CFU), specified by unique profiles of gene expression under the control of master genes and transcription factors, in response to different hemopoietic growth factors or combinations thereof[1]. Progenitor expansion is adjusted to the turnover rate of the respective circulating forms of each lineage, so that relatively stable numbers of red cells, platelets and leukocyte subpopulations are replaced every day, enabling us to determine a range of "normal" blood cell counts, which may widely differ from one lineage to the other[1,3].

The original *in vitro* studies, which led to the purification and ultimately to cloning of a variety of colony-stimulating factors (CSF) of various nonlymphoid sources, endowed with selectivity for macrophage (M-CSF, or CSF-1), granulocyte (G-CSF), or granulocyte-macrophage (GM-CSF) progenitors, had suggested that hemopoiesis in steady-state conditions was driven by CSF-like molecules[3]. From this assumption one would predict that disruption of signaling by CSF-like molecules would entail profound deficiency in circulating leukocytes. This view must now be qualified, however, in view of the persistence of normal granulopoiesis in mice lacking the functions of GM-CSF and IL-3, two major CSF species[13]. Not all CSF, however, are irrelevant to steady-state granulopoiesis, as IL-5 seems necessary for normal production of eosinophils[9,13-15], G-CSF for that of neutrophils and M-CSF for that of macrophages[1]. Thrombopoietin and G-CSF, originally identified as CSF with lineage-selectivity for megakaryocytes/platelets and neutrophils, respectively, have been further characterized as multilineage regulators with complex actions, thereby overstepping the original boundaries of their function[1,3]. Therefore, while much remains to be learned about the intrinsic processes that drive definitive hemopoiesis in steady-state, it is likely that at least some CSF cytokines contribute to hemopoiesis in exceptionally demanding conditions, by mediating the actions of extrinsic factors linked to homeostatic disturbances or environmental changes on bone-marrow.

Increased demand on the bone-marrow imposed by systemic challenges, unlike regeneration of the entire hemopoietic environment[16], elicits lineage-selective responses, which may be short- or long-lived: For instance, hemorrhage and chronic hypoxemia are met with compensatory production of erythrocytes[17]; in other examples, bacterial infection elicits adaptive increases in neutrophil leukocytes[4,5,11], and helminth infection or allergic disease induce eosinophilia[9,14,18-20].

Importantly, the critical elements in these adaptations of bone-marrow to a transient stress are lineage-committed progenitors, rather than the HSC endowed with

self-renewing and long-term repopulating potential. This makes biological sense, since progenitors are closer than stem cells to terminally differentiated, functional blood cells, and the physiologically relevant increase in circulating blood cells will be faster, because it will require less rounds of cell division. By contrast, HSC, as a rule, are protected from such transient challenges for a good reason, since infection at least may severely impair their function[11].

GM-CSF and interleukin (IL)-3 may be more relevant to the stress (or emergency) myelopoiesis in systemic microbial infection[4,5,15], and, in the more restricted context of helminth infection and allergic disease, IL-5 plays an important role for its selectivity to the eosinophil lineage[9,14,19].

Importantly, however, in the case of neutrophil or eosinophil granulocytes, proliferative and maturation-promoting effects of these CSF on production are only part of their contribution to the adaptive hematological responses, since they also have important mobilizing effects on the reserve pool associated with bone-marrow and other sites, and they further extend the lifespan of selected hemopoietic lineages outside bone-marrow, thereby increasing the total number of cells belonging to these lineages in the periphery, and decreasing their turnover by a lineage-selective reduction in cell death rates[7]. The consistently positive action of the same CSF at multiple steps in the life cycle of granulocytes highlights the integration of these proliferative and non-proliferative cytokine effects, which translates in physiologically meaningful outcomes.

It is important that these granulopoietic/mobilizing/antiapoptotic cytokines are not restricted to the bone-marrow compartment, but are often produced by multiple cell types in the context of specific adaptive (specific) as well as innate (nonspecific) immune responses at distant sites. Nevertheless, cytokines acting on bone-marrow targets act early in this sequence, and due to the amplification of their effects through multiple rounds of cell division, they have long-lasting effects.

In the context of allergic disease or helminth infection, IL-5, the lineage-specific cytokine required for both constitutive and stress eosinopoiesis, is secreted in different contexts by different cells, especially by activated, allergen-specific, Th2 lymphocytes[1,9,14,15], and could contribute in various ways to the effects of allergen challenge. Recognition of allergen at the challenge site by TH2 lymphocytes, which subsequently secrete IL-5, is one way to couple allergen recognition in the peripheral sites to generation of a stimulus within the bone-marrow. Other possibilities include production of IL-5 by mast cells following recognition of allergen by specific IgE bound to FcεRI in the mast cell surfaces[15,20]. Secretion of IL-5 inside bone-marrow by lymphocyte populations[21] might also contribute, although it is unclear at present whether these would necessarily be conventional T lymphocytes, requiring MHC restricted allergen presentation by dendritic or B cells.

THE MODES OF OPERATION OF CYTOKINES AND OTHER EXTRINSIC REGULATORS OF BONE-MARROW FUNCTION

Cytokines can transduce the effects of immune reactions on the bone-marrow, by one of two ways: (1) systemic diffusion of the cytokine itself, from the inflammatory site to bone-marrow through the circulation; and (2) selective homing of cytokine-producing cells to the bone-marrow, followed by local cytokine production.

In the first case, the cytokine stimulus is widespread, but the response remains restricted because the relevant target cells are concentrated in bone-marrow or, if present elsewhere, are presumably absent or dormant. In the second case, such a systemically diffusible stimulus is not necessary or even relevant, since the effect of immunity on granulopoiesis is elicited through a local accumulation of cytokine-producing leukocytes inside the bone-marrow, which only activate the relevant target cells in their neighborhood. Both mechanisms depend on the stimulus not being constitutively present, or effective, but becoming so in the wake of allergen challenge.

These alternatives have clearly distinct counterparts in an experimental setting: In the first case, bone-marrow effects can be elicited by intravenous transfer of plasma from the appropriate donors to naive recipients[22], and effects of this transfer will be restricted to the bone-marrow as long as there are no responsive targets elsewhere; in the second, transfer of leukocyte populations capable of homing to the bone-marrow compartment will be sufficient, and responses will both be limited to the bone-marrow compartment, and associated with the physical presence of the transferred leukocytes in this compartment[23].

It should be noted that these various possibilities are not mutually exclusive, but may better describe events at different time points. This is probably relevant to the sequence of events elicited by allergen exposure of sensitized mice ("challenge"), thought to represent chronic processes that underlie allergic diseases, especially asthma[12,24], as discussed below in the context of eosinophil production, which is the prime target of IL-5 actions.

It is also important that CSF-like molecules are just a small fraction of the cytokines that might be influencing bone-marrow responses, which is defined by its ability to directly stimulate hemopoiesis. A much larger number of cytokines may be unable to act as primary hemopoietic stimulus, but remain quite effective in modifying the actions of primary stimuli to achieve particular effects. In the case of the eosinophil lineage, IL-5 is the best characterized (and highly selective) primary stimulus[9,15,20]; however, a number of cytokines discussed below, including eotaxin/CCL11, IL-13 and IL-17, do not stimulate directly eosinopoiesis, but interact with IL-5 to

achieve quite different outcomes[25,26].

Another important feature of cytokine-coordinated processes is the potential for interactions involving multiple partners. These are, in some cases, other cytokines as mentioned above; however, they may also include noncytokine mediators of inflammation such as proinflammatory or antiinflammatory lipid mediators, hormones, or vitamins. In the context of therapy, drugs or immunoregulatory leukocyte subpopulations may become partners for novel interactions. Generally speaking, the actions of a given cytokine may be only understood in context, which encompasses immunoendocrine and immunopharmacological interactions, in addition to cytokine network interactions.

THE EXPERIMENTAL ANALYSIS OF ALLERGEN-INDUCED EOSINOPHILIA

Eosinophils, a minor subset of circulating leukocytes, remain better understood in their contributions to tissue injury in allergic disease such as asthma[27,28], than in their presumably beneficial actions in host defense and tissue repair[9,14,15,29,30]. Nevertheless, eosinophils are very interesting cells, which produce a large number of specialized (mostly cationic, or "basic") proteins, a complex mixture of lipid mediators, and an impressive number of cytokines, which overstep the boundaries of the conventional TH1 and TH2 "profiles". In addition, eosinophils aided by antibody are capable of killing some tumor cell targets, and, at high effector/target ratios, some larval stages of worms[19]. It is both biologically puzzling and therapeutically serendipitous that eosinophil-depleting interventions in experimental models as well as in chronic treatments have no consistent adverse effects. Hence, the damage they could cause in people with a variety of diseases is prevented by such treatments, but no obvious function for eosinophils in an otherwise healthy subject is unveiled through eosinophil depletion[29]. This does not necessarily prove that eosinophils have become obsolete in the course of evolution; it nevertheless suggests that beneficial functions for eosinophils might be relevant in very specific conditions which have not yet been addressed in these previous studies. Two such examples in the recent literature are a beneficial role for eosinophils in liver regeneration[31] and a role for eosinophils in the recruitment of other leukocyte classes in response to CCL11[32].

Eosinophils are very suitable for the experimental analysis of stress granulopoiesis in mice, for a number of reasons.

Reliable identification and efficient detection of eosinophils

In mice and humans, eosinophils are easy to recognize and to detect in tissues, by a combination of surface marker expression and morphological criteria, including cytochemical reactions. Although nuclear morphology is not identical between human and mouse eosinophils, they are polymorphonuclear leukocytes presenting segmented, thick bands of chromatin when mature[9,14,15,33]. In both species, they contain numerous cytoplasmic granules of various sizes, and in the mouse a coarse type of granule, displaying the characteristic affinity for the acidic dye eosin, also stains positive in the cytochemical reaction for eosinophil peroxidase (EPO), which yields a brown color because of precipitated diaminobenzidine product formed in the presence of exogenous H_2O_2[34]. Murine EPO, unlike its homologues in other species, including humans[35], is resistant to cyanide, which makes this reaction a very useful marker for mouse eosinophils, as distinct from mouse neutrophils. Experimentally, the expression of a characteristic array of surface markers, including the receptor for the lineage-selective chemokine, CCL11 (or eotaxin-1), CCR3[36], as well as the cell surface lectin Siglec-F or Siglec-8[9], makes it easier to monitor the presence of eosinophils in cell suspensions by flow cytometry.

Quantitative changes can be accurately induced and monitored

The numbers and even the presence of eosinophils in individual animals can be manipulated conveniently in various murine models. Allergic sensitization and challenge, helminth infection, IL-5 overexpression and/or administration, IL-9[37] and more recently IL-33 infusion[38], have all been reported to induce eosinophilia in mice[9,14,22,39]. Eosinopenia, the opposite state, can be induced in variable degrees by a variety of neutralizing antibodies to IL-5[22,40] or by induced mutations, including selective deletion of an internal autoamplification site in the promoter of the GATA-1 transcription factor, through which the coding regions of the GATA-1 gene remain functionally intact and sufficient for differentiation of erythrocytes and platelets, while eosinophil production, which requires an early autoamplification step, fails on a permanent basis[41]. Even more conveniently for the experimenter, this model is suitable for reconstitution, since mature eosinophils can be transferred from wild type (BALB/c) control donors in high purity[32]. Alternative models, based on selective and conditional elimination of eosinophils by genetic engineering, have provided important insights in other experimental models[27,28,42]. It is reassuring that no obvious damage to the organism is associated with eosinophilia or eosinopenia per se, as documented even in IL-5 transgenic models. By contrast, damage to heart and nervous tissue, and extremely high eosinophilia, not induced by an external agent, coexist in humans with the so-called hypereosinophilic syndromes; there is, however, extensive evidence that, in these conditions, eosinophils are functionally activated and possibly abnormal[9,20]. In murine models[39], by contrast, no damage secondary to the induction of eosinophilia is likely to confound the interpretation of results.

Stimuli and procedures have a high degree of selectivity

Usually, marked changes in eosinophil counts occur in bone-marrow, spleen or blood without significant changes in total cell counts. This apparent selectivity is

due to eosinophils being a minority[9,14], amounting to 3% or less of circulating leukocytes in noninfected, nonallergic humans, for instance, while most other leukocyte populations are much larger (compare with up to 70% neutrophils in human blood). To reach significance, a much larger change in other leukocyte populations would be needed, because random fluctuation is larger in this case. Eosinophils have a specialized growth factor (IL-5), and differ from other leukocyte types in responses to other cytokines and mediators of inflammation, including rather selective chemoattractants, such as CCL11[33] and the cysteinyl-leukotrienes (CysLT)[43]. All of these differences contribute to the relative selectivity of the effects observed. Nevertheless, some stimuli, such as GM-CSF, elicit major responses in several hemopoietic lineages at once, including eosinophils and neutrophils[44]. It is relevant, in this context, that GM-CSF and IL-5 receptors, although distinct in their composition, share an essential signaling component, the common β chain (βc[13,44]). This subunit is also found in IL-3 receptors, although in mice there is evidence for a separate IL-3 receptor lacking βc[13]. Eosinophils and neutrophils present GM-CSF and IL-3 receptors bearing βc, while eosinophils (and basophils[42]) have IL-5 receptors, unlike neutrophils[8,13]. This implies that even though GM-CSF, IL-3 and IL-5 can stimulate eosinophil production through similar signaling pathways (mediated by βc), IL-5, unlike GM-CSF and IL-3, should not directly induce neutrophil production.

THE SPECTRUM OF CHALLENGE EFFECTS ON THE EOSINOPHIL LINEAGE INSIDE BONE-MARROW

We have first described the upregulation of eosinophil production in murine bone-marrow in mice sensitized and challenged with allergen in the airways. In this murine model of allergic eosinophilia, the critical stimulus is specific allergen challenge in the airways[22] or in alternative sites[45], and the major outcome is an increase in bone-marrow eosinophil-lineage cells (eosinophil peroxidase positive, or EPO^+, cells) in bone-marrow harvested 24 h after challenge, which is taken as direct evidence of allergen-induced eosinophilia of the bone-marrow *in vivo*. To keep a focus on bone-marrow events, we will not discuss here extramedullary effects of allergen challenge, such as the accumulation of eosinophil progenitors in the lungs[46] and the large increase in eosinophils in the spleen[47]. Nevertheless, these are interesting in themselves and share important mechanisms with events in bone-marrow[47].

Challenge-induced bone-marrow eosinophilia can be fully prevented in mice made specifically tolerant to the allergen before sensitization and challenge, as well in recipients of splenic T cells from these tolerized donors[48]. It is important, however, that the tolerance induction oversteps the boundaries of the original phenomenon, as tolerance-induced changes also affect neutropoiesis and therefore extends to another lineage[48].

This sensitization/challenge experimental setting provides a wealth of experimental opportunities, which have been explored in recent years. Because of the rapidity with which events in the airways translate into distant consequences in the bone-marrow, we hypothesized that a mediator released in the circulation would account for communication between the sites of challenge and of eosinopoiesis. Plasma transfer experiments from sensitized/challenged donors to naive recipients did support this view[22].

While *in vivo* observations suggest the relevance of the phenomenon, important information was provided by *ex vivo* protocols, defined as the *in vitro* analysis of changes induced by previous interventions *in vivo*. The outcome of *in vivo* interventions and the associated *ex vivo* observations is summarized in Table 1. In addition to rapidly inducing eosinophilia of bone-marrow *in vivo* (24 h), challenge also increases the magnitude of the responses of bone-marrow cells to IL-5. This effect was described as "priming" because it takes place *in vivo*, during the 24 h that follow challenge, but it remains undetected until cells are exposed to IL-5 in culture for several days - hence it corresponds to a silent change in developmental potential that is unveiled by subsequent IL-5 exposure[22,45]. It is analogous, but not identical, to priming for other cellular responses by exposure to IL-5 itself[49], since it distinguishes between *in vivo* allergen-challenged and -unchallenged sensitized mice, which do not necessarily differ in their circulating IL-5 levels[22]. The endpoint that defines priming is *in vitro* differentiation of precursors that had been exposed *in vivo* to allergen and presumably to newly released IL-5 as well. Nevertheless, these precursors are not IL-5-autonomous, since they do not complete differentiation in culture if exogenous IL-5 is not added[22]. This apparent paradox suggests that even though endogenous IL-5 has been shown be present *in vivo* in the bone-marrow after challenge[21], it is not sufficient to sustain eosinophil production over a week's culture.

Importantly, priming is a positive phenomenon: It rapidly increases the magnitude of the responses to IL-5 as well as to other eosinopoietic stimuli, such as IL-3[22]. Therefore, it is assumed to contribute to the eosinophilia of allergic disease, rather than to oppose it. It is detectable as early as 24 h and as long as one week after challenge of sensitized mice.

Priming, however, is paralleled by a distinct *ex vivo* effect[50], which we call immunoregulation of pharmacological responses, because it reduces the magnitude of the subsequent responses of cultured bone-marrow to some drugs, as well as to exogenously provided mediators and cytokines. In contrast to priming, which changes the response to the primary stimulus (IL-5), immunoregulation of pharmacological responses attenuates the response to secondary stimuli, such as nonsteroidal antiinflammatory drugs (NSAID) and proallergic cytokines, all of which require IL-5 to be effective. Hence, priming upregulates a core response to IL-5; by contrast, immunoregulation restricts

Table 1 The spectrum of GC-dependent effects on bone-marrow eosinopoiesis

In vivo treatment	Bone-marrow effects						Systemic factors		Ref.
	In vivo bone-marrow Eosinophilia	*Ex vivo* effects on response to GM-CSF (CFU counts) in bone-marrow culture		*Ex vivo* effects of challenge on responses to IL-5 or CysLT in bone-marrow culture			GC measurements and interventions targeting GC		
		Total	Eosinophil	Priming	Maturation of eosinophils in culture	Regulation of CysLT responses	Blockade by GC targeting	Plasma corticosterone	
None	Baseline	NA	NA	NA	Complete	NA	No effect	Baseline	[36,52]
Dexamethasone (5-20 mg/kg)	Baseline (BALB/c)	Increase	Increase	Primed by 24 h of injection	Incomplete, rescued by PGE2, anti-α4 integrin antibody	NE	RU486	NA	[51,36]
	Increased (C57BL/6)	Increase	NE	NE	NE	NE	RU486	N. A.	[53]
Surgical trauma	Increased (BALB/c) by 24 h of trauma	NE	NE	Primed by 24 h of trauma	Complete	NE	RU486 Metirapone	Stress level by 24 h of trauma	[52]
Sensitization and challenge	Increased (BALB/c, B6, BP-2) by 24 h of challenge	No significant increase	Increased by 24 h of challenge	Primed by 24 h of challenge	Complete	Attenuated by 24 h of challenge	RU486 Metirapone (eosinophilia, priming)	Stress level by 24 h of challenge (BALB/c), TNF-α induced	[22,25,45, 47,50,54,70]
Oral tolerance induction, sensitization and challenge	Increase prevented (BALB/c, BP-2) by 24 h of challenge	Increased by 24 h of challenge	Increased by 24 of challenge	Priming prevented (BP-2) by 24 h of challenge	Complete	NE	NA	NE	[48]

CFU: Colony-forming unit; NE: Not examined in the study; NA: Not applicable to the study.

a peripheral response to modifiers of IL-5 activity. While priming is assumed to promote an eosinophilic response, immunoregulation should, in principle, restrict further expansion of eosinophil numbers by exposure to other stimuli. These two effects do not cancel each other, but may interact, depending on the precise stimulation context and on their relative timing.

Indomethacin and aspirin, two NSAID with biochemically distinct mechanisms of action, proved stimulatory not only for IL-5-driven eosinopoiesis in bone-marrow culture, but for colony formation by myeloid progenitors of several lineages as well[50]. Unexpectedly, however, when bone-marrow from sensitized and challenged mice was studied, the ability of both NSAID to enhance eosinopoiesis was lost[50], and, depending on the experimental conditions, NSAID can even become inhibitory in this assay (manuscript in preparation). Therefore, immunoregulation of pharmacological responses comprises two aspects: (1) attenuation of the effectiveness of NSAID as enhancers of eosinopoiesis; and (2) inversion of its effects, leading to active suppression of eosinopoiesis when NSAID are present. Because pharmacological responses are usually not assumed to depend on the immune status of the organism, this apparently paradoxical observation has theoretical interest in itself. In its original description[50], no physiological relevance was ascribed to it. More recent results, as detailed below, substantially increased the scope of this effect and highlighted its potential to modulate eosinophilia in a biologically more relevant context.

The twin phenomena of priming and pharmacological immunoregulation highlight two important features of extrinsic control of bone-marrow: (1) changes can be both silent and durable, as in priming, thereby accounting for effects that may become visible only in the long-term; and (2) the apparent paradox of a drug response that depends on the immune status of the organism - challenged *vs* unchallenged mice - reflects the mechanism of action of the drug as well as the inflammatory events elicited by challenge. Both priming and immunoregulation remain silent effects, until they are unveiled by the appropriate stimuli (exposure to IL-5, in the first case; or to NSAID in the presence of IL-5, in the second). In both cases, challenge changes the properties of the target cell.

THE CENTRAL ROLE OF GLUCOCORTICOIDS IN EXTRINSIC REGULATION OF BONE-MARROW EOSINOPOIESIS

A central contribution of glucocorticoids (GC) to extrinsic bone-marrow regulation was first reported in a pharmacological setting, following exposure to dexamethasone, both *in vivo* and *in vitro*[51]. Subsequent experiments in a sterile trauma model indicated that stress-induced GC (corticosterone, in mice) selectively induced bone-marrow eosinophilia in the absence of

specific immune responses[52]. Finally, an essential role for corticosterone in allergen-induced bone-marrow eosinophilia was also demonstrated in a sensitization/challenge model[45], which necessarily involves specific immunity. Hence, evidence of a link between GC and bone-marrow eosinophilia was consistently provided by experiments ranging from pharmacological through physiological to immunological settings, which are also summarized in Table 1. This coherence of effects is to be expected from the well-established fact that GCs, synthetic or natural, act through the same receptor, which is blocked by RU486 (mifepristone[45,51-53]).

Dexamethasone did not induce bone-marrow eosinophilia *in vivo* in our original study, which used mice of the BALB/c background; however, it did prime bone-marrow for strongly enhanced responses to IL-5 *ex vivo* over a period of from 24 h[51] up to 4 wk after injection (manuscript in preparation); more recently, however, an important difference between strains of distinct backgrounds was observed for this drug effect, since bone-marrow eosinophilia was observed in C57BL/6 mice injected with dexamethasone, 24 h after injection, unlike BALB/c controls[53]. In both BALB/c and C57BL/6 mice, dexamethasone primed bone-marrow for increased eosinophil production in IL-5-stimulated cultures; dexamethasone did not replace IL-5 as a primary eosinopoietic stimulus, but greatly enhanced its effectiveness. However, dexamethasone significantly modified IL-5 effects, since a large fraction of the eosinophils produced in dexamethasone-exposed BALB/c cultures were cytologically immature and formed extensive homotypic aggregates[36,51], none of which had been observed in preceding studies of BALB/c[22,45] or C57BL/6[54] sensitized/challenged mice. Further studies[36] demonstrated the ability of PGE2 to synergize with dexamethasone in promoting terminal cytological maturation of these eosinophils in BALB/c bone-marrow cultures. Because neutralizing antibodies to VLA-4 (CD49; $\alpha4\beta1$ integrin) were able to dissociate the homotypic aggregates formed in dexamethasone-exposed cultures, leading to an increased recovery of fully mature eosinophils, we hypothesized that homotypic aggregation interfered with terminal maturation, and that release from aggregates allowed terminal maturation to proceed. Accordingly, PGE2 was shown to dissociate the same aggregates, through an effect on $\alpha4\beta1$ integrin expression[36].

By contrast, in the trauma model[52], the physiologically relevant GC, corticosterone, was elevated to stress levels 24 h after surgery and selectively induced eosinophilia in the bone-marrow, as well as primed for increased IL-5-dependent eosinopoiesis. These effects of trauma-induced GC were long-lasting, and significant at least for two weeks after surgery[52]. The direct contribution of glucocorticoids in this model was documented by blocking with RU486, as had been previously done with dexamethasone[51], and confirmed by two other independent approaches (metirapone treatment and adrenalectomy followed by trauma after a recovery period).

Unlike the response to dexamethasone injection, it is likely that the response to trauma adds to the GC surge other variables related to cell injury and innate immunity. One important such variable, is tumor necrosis factor-α (TNF-α), which may interact with corticosterone so as to modify its actions, in a way consistent with the differences observed between the pharmacological (dexamethasone) and the physiological (trauma) models, especially in the induction of bone-marrow eosinophilia and on cytological maturity of the eosinophils.

Finally, we recently demonstrated that the eosinophilia induced by challenge in sensitized mice involves endogenous GC[45], which are induced by a product of immune cell activation, TNF-α, because: (1) eosinophilia is abolished with equal effectiveness by RU486 or by anti-TNF-α neutralizing antibody; and (2) a corticosterone surge, reaching stress levels, is observed in wild-type controls, with or without RU486-pretreatment, but not in TNF-α type Ⅰ receptor-deficient (TNFRI-KO) mice.

Challenge-induced eosinophilia is sustained by IL-5 acting *in vivo*[21,22]; by contrast, priming requires endogenous GC to act *in vivo* to prime for an increased *ex vivo* response to IL-5 upon subsequent culture[45]. Although IL-5 has usually been considered the target of changes initiated by challenge, there is evidence that responses to IL-5 are self-limiting themselves, since exposure to IL-5, IL-3 or GM-CSF, presumably acting through βc, was shown to down-regulate IL-5Rα chain expression[55], thereby reducing IL-5 binding and strength of stimulation. Similar observations were reported with other extrinsic regulators, such as all-trans retinoic acid, which suppresses expression of IL-5Rα in culture of human hemopoietic cells[56]; in murine eosinopoiesis, the effects of all trans retinoic acid are effectively blocked by GC (Xavier-Elsas *et al*, submitted).

Together, these observations, summarized in Table 1, are consistent with the reported ability of TNF-α as well as IL-1β, both major inflammatory cytokines, to mediate, in animal models, an immunoendocrine response to tissue damage with stress-levels of adrenal GC[57]. They are equally consistent with the reported link between elevated levels of cortisol in humans subjected to stress and an increase in the frequency and severity of allergic reactions[58,59]. Against this background information, the extrinsic upregulation of bone-marrow eosinophilia by dexamethasone, trauma and allergy in our own studies is better explained as a paradoxical stimulatory effect of GC on progenitors and precursors of the eosinophil lineage. While this may go against the predominant view of GC effects in allergy[57,60-62], it is a reproducible effect with pathophysiological implications[58,59,63-65], which may appear less paradoxical as a result of its interaction with the 5-lipoxygenase (5-LO) pathway of arachidonate metabolism, as detailed below.

THE MULTIPLE ROLES OF 5-LO IN BONE-MARROW: SOLVED, UNSOLVED AND NOVEL QUESTIONS

The 5-LO pathway, which produces leukotrienes (LT), has been intensively studied over three decades in the

context of allergic disease[66,67]. While its involvement in the functional abnormalities of the airways in asthma is well-established, its roles in extrinsic regulation of bone-marrow remain incompletely explored, even though bone-marrow is believed to be central to chronic inflammation in asthma[12,24]. LT, especially CysLT (a class comprising LTC4, LTD4 and LTE4), besides making important contributions to asthma pathophysiology, have significant pharmacological effects on hemopoietic cells[68,69]. Such effects are of special interest in the case of eosinophils, which both produce and respond to LT[43], and play important roles in allergic disease. Type 1 CysLT receptors (CysLT1R) play important roles in the pathophysiology of human and experimental asthma, and CysLT1R antagonists, such as pranlukast, zafirlukast and montelukast, are currently approved for the treatment of asthma[47,66,67]. CysLT1R are expressed in several cell populations in the bone-marrow[68], and CysLT were shown to enhance colony formation by progenitors of different myeloid lineages in addition to eosinophils[69].

The stimulatory effect of NSAID on eosinopoiesis is of special interest not only because it is subject to immunoregulation of pharmacological responses, but because of what it tells us about the roles of COX and 5-LO in bone-marrow regulation.

NSAID, which block the cyclooxygenase (COX) pathway, were originally tested in the context of the effects of prostaglandin E2 (PGE2), a COX product, in murine bone-marrow culture. Eosinopoiesis is significantly suppressed by exogenously added PGE2 in bone-marrow cultures established from allergen-challenged mice, as well as from unchallenged controls. This suppressive effect of PGE2, which is unaffected by allergen challenge, is not surprising in itself, because nonselective inhibitory effects of PGE2 on hemopoiesis *in vitro* have long been known[36], and suppression of eosinopoiesis would seem to be just one particular example of this general phenomenon. On the other hand, if NSAID were working solely as COX inhibitors, they should be ineffective against exogenously added PGE2, which bypasses the COX pathway to act directly on PGE2 receptors. However, we showed that NSAID prevent the suppressive effects of PGE2 on eosinopoiesis, and further stimulate eosinophil production strongly (in this case, the eosinophils are cytologically mature)[70]. This rules out a mechanism involving only COX inhibition, which cannot protect against preformed COX products. Furthermore, blockade of the 5-LO pathway by genetical or pharmacological approaches abolishes the effectiveness of NSAID in promoting eosinopoiesis, which implies a 5-LO-mediated mechanism, quite distinct from simple COX inhibition. This alternative mechanism was shown to depend on CysLT, endogenously produced in bone-marrow culture, as evidenced by its absence in LTC4 synthase-deficient mice, and by its blockade by CysLT1R antagonists and CysLT1R deletion. In support of this view, exogenously added CysLT strongly stimulate eosinopoiesis, even in the absence of functional 5-LO or LTC4 synthase[70].

Importantly, this same CysLT-mediated mechanism was subsequently shown to underlie the stimulatory effects of eotaxin/CCL11 and IL-13, two cytokines central to allergic inflammation, on eosinopoiesis in naive bone-marrow culture[25]. Again, both depend strictly on functional 5-LO and CysLT1R to enhance eosinophil production, and both lose effectiveness when bone-marrow from sensitized-challenged mice is used.

It is clear, therefore, that extrinsic regulators of bone-marrow eosinopoiesis may be subject to immuno-regulation (NSAID, proallergic cytokines) or not (PGE2), depending on their mechanism of action.

Since IL-13 and eotaxin are produced during allergic episodes and present systemic effects[15,33,40], this suggests that CysLT in bone-marrow are proximal elements in a chain of events started by a distant allergic reaction, and therefore might play a role in the strong upregulation of eosinophil production following challenge. Consequently, one might predict that targeting CysLT production or signaling with drugs currently in use (respectively zileuton for production and montelukast and its analogues for signaling), would not only be beneficial in attenuating local allergic symptoms, but also in preventing increased eosinophil production. Such an effect of pranlukast has been previously reported in humans[71].

In ovalbumin-sensitized mice, we have observed the complete blockade of challenge-induced eosinophilia of the bone-marrow using both the leukotriene synthesis inhibitor diethylcarbamazine (DEC)[54] and 5-LO-activating protein inhibitor MK-886[47], which prevent production of CysLT; the same effect was observed with montelukast, which blocks CysLT1R[47]. Consistently with this hypothesis, DEC had no effect in mice lacking functional 5-LO. Together, this evidence supports an essential role for CysLT in challenge-induced eosinophilia, similar but distinct from that previously attributed to endogenous GC.

Further insight on the underlying cellular mechanisms is provided by the effects of DEC. Interestingly, DEC requires not only 5-LO to be effective[47] but inducible NO synthase (iNOS) as well[54]. This enzyme, which produces large amounts of NO in the course of cellular immune responses to a number of intracellular pathogens, had already been shown to be required for the suppressive effects of PGE2 on bone-marrow eosinopoiesis[72]; more recently, it was shown to account for similar effects of α-galactosylceramide (α-GalCer; an anticancer agent and immunomodulator)[23] and IL-17 (a powerful proinflammatory cytokine)[26] on bone-marrow. These observations, therefore, establish DEC as a pharmacological link between 5-LO and iNOS in bone-marrow, as discussed below. It should be noted that GC powerfully suppress iNOS expression[72], and this underlies their ability to block the suppressive effect of PGE2: *In vitro*, when iNOS expression is blocked by dexamethasone, this GC interacts with PGE2 to increase the production of mature eosinophils in culture[36], a somewhat unexpected interaction between an anti-inflammatory drug and a proinflammatory mediator.

It is therefore important that CysLT can counteract the effects of both IL-17[26] and α-GalCer[23] on eosinopoiesis, just as CysLT counteract those of PGE2[70]. Like GC,

therefore, CysLT target the iNOS-CD95-dependent proapoptotic mechanism that suppresses eosinopoiesis, as part of their eosinophilia-promoting actions.

Together, the available evidence suggests that challenge-induced eosinophilia in the bone-marrow is associated with both iNOS suppression (by GC) and 5-LO-mediated mechanisms; by contrast, its prevention is associated with iNOS-mediated mechanisms and blockade of 5-LO. It is therefore important to understand how these regulatory and effector elements (GC, 5-LO, iNOS) relate to each other.

If immunoregulation of responses to NSAID involved modulation of the COX pathway as opposed to the 5-LO pathway, responses to exogenously added CysLT in the bone-marrow would not depend on the immune status of the mouse. However, just like for NSAID, responses to LTD4 are strongly immunoregulated in murine bone-marrow (manuscript in preparation). This suggests that challenge not only requires CysLT to increase eosinophil production, it also profoundly attenuates the effectiveness of CysLT, thereby limiting the magnitude of responses to drugs and cytokines which are mediated by endogenous CysLT.

Because the effects of challenge on bone-marrow are counteracted with similar effectiveness by blockade of endogenous GC signaling, and by blockade of CysLT1R signaling, this raises the issue of the relationship of endogenous GC to CysLT in the context of sensitization and challenge.

While the similar actions of endogenous GC and CysLT might seem unrelated or even incompatible, recent studies point to a critical partnership of these mediators *in vivo*. This prompted us to address in the following section how these quite dissimilar classes of extrinsic regulators might work together to induce eosinophilia in murine bone-marrow, and to further limit the magnitude of this response to challenge through subtle regulatory mechanisms.

MAKING SENSE OF AN UNLIKELY PARTNERSHIP: CHALLENGE-INDUCED EOSINOPHILIA AS A SELF-LIMITING PROCESS STARTED BY GC AND AMPLIFIED BY CYSLT

The observations summarized above may appear paradoxical in several respects: (1) GC are usually thought of as antiallergic agents, not as promoting allergy; (2) GC are believed to suppress the generation of lipid mediators from arachidonate metabolism (eicosanoids) and should accordingly prevent the generation of CysLT; (3) even though GC are essential to the effects of challenge on the bone-marrow, dexamethasone alone cannot reproduce all of these effects; (4) GC (anti-allergic agents) and CysLT (proallergic agents) seem to elicit the same outcome - increased eosinophil production - and therefore constitute a highly unlikely couple; and (5) the effects of CysLT, furthermore, are attenuated after challenge, and may even become suppressive, in a clear inversion of the original signal provided by these mediators.

GC and CysLT form indeed an odd couple: GC are widely used as anti-inflammatory agents and for the long-term maintenance in asthma control; by contrast, CysLT account for some of the most visible manifestations of asthma and allergy, and CysLT antagonists are useful for asthma control. So GC and CysLT would be expected to be natural antagonists, not partners. However, the eosinopoiesis-enhancing effects of dexamethasone are observed at lower concentrations that its anti-inflammatory and anti-allergic effects[51], and are compatible, in terms of glucocorticoid activity, with the GC surges associated with acute stress[45,52]. So, GC promotion of eosinopoiesis by dexamethasone might be dose-dependent and self-limiting over time.

This priming effect of GC is reproduced by surgical trauma in the absence of allergen sensitization[52], and is therefore independent of underlying allergic processes. When the relevant GC, corticosterone, is released by challenge of sensitized mice, this release depends on TNF-α type 1 receptors, and is therefore part of the nonspecific host response to aggression, mediated by proinflammatory cytokines[45]. The effect of challenge seems to last about one week[54], although surgical trauma has a longer-lasting impact on bone-marrow[52], and dexamethasone may have a priming effect demonstrable in bone-marrow culture as long as one month after a single injection (manuscript in preparation). Our interpretation is that the duration of GC effects is significantly curtailed by factors operating *in vivo* during trauma or allergic challenge, and, in a sense, this makes the extrinsic regulation of bone-marrow eosinopoiesis by allergen challenge a self-limiting process.

On the other hand, the results of complete prevention of challenge-induced eosinophilia by a variety of interventions that target CysLT production or signaling (DEC, MK886, 5-LO deficiency; montelukast) can only be understood if one assumes that despite the elevation in endogenous GC levels induced by challenge[45], the 5-LO pathway is operative and CysLT are produced. Resistance of CysLT to GC even at therapeutic levels has been reported in human studies[67] and underlies the rationale for using antileukotrienes as complementary to GCin asthma control[62,66,67].

If both endogenous GC and CysLT are present *in vivo* after challenge, what is their relationship? They might be present simultaneously, but at different, unconnected sites and therefore act independently of each other; alternatively, they might be coupled. Prevention of challenge-induced eosinophilia by GC or by CysLT blockade to the same extent might seem to argue against either having an independent effect on bone-marrow, since one would logically expect an additive effect of blocking both targets, which is not observed. However, bone-marrow of naive mice[70], or from sensitized mice pretreated with RU486 before challenge (manuscript in preparation), does respond to CysLT in culture in the absence of exogenously

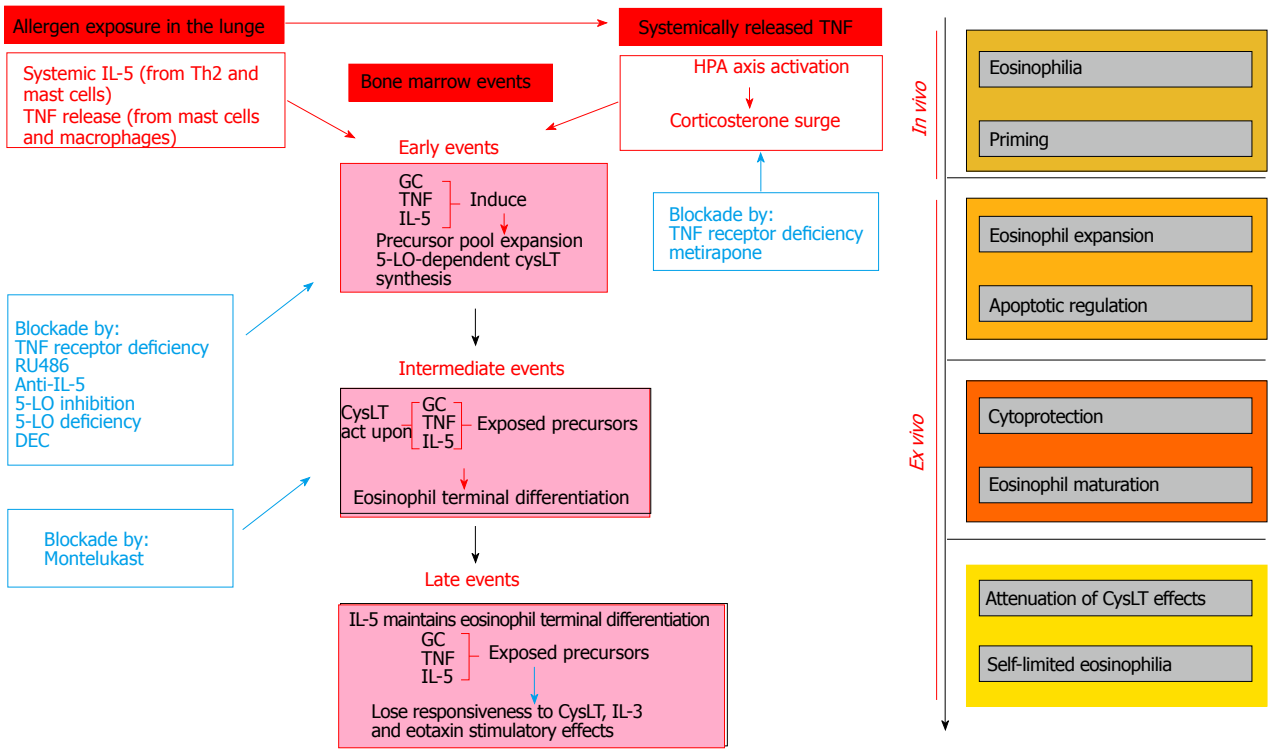

Figure 1 Events outside and inside bone-marrow following allergen challenge. The sequence of critical events in the lungs, endocrine system and bone-marrow is outlined on the left as a flow chart, and their impact on the establishment of bone-marrow eosinophilia is depicted on the right as a timeline. On the left, we outline the contributions of cytokines (IL-5, TNF-α), adrenal GC hormones, and CysLT at early, intermediate and late phases after challenge, as have been characterized by genetical, immunological and pharmacological tools in bone-marrow culture (*i.e.*, *ex vivo*; refs. provided in Table 1). Events promoting allergic inflammation are shown in pink boxes; interventions opposing allergic inflammation are shown in light blue boxes. Systemic events preceding the local bone-marrow response (left side, lungs; right side, endocrine system) are shown in red boxes. RU486 (mifepristone) is a blocker of GC receptor; metirapone is an inhibitor of adrenal GC biosynthesis. The combination of IL-5, TNF-α and adrenal GC is considered to be critical for the entire sequence of events in the bone-marrow, due to long-lasting effects of exposure during the initial 48 h of culture[51]. CysLT act downstream from GC[45,47] in the same sequence of events. Challenge promotes expansion of eosinophil precursors and their maturation in the presence of CysLT *in vivo*, but also attenuates responses to CysLT during subsequent exposure *ex vivo*, thereby limiting the magnitude of the resulting eosinophilia (represented in shades of orange at the right). TNF-α: Tumor necrosis factor-α; IL: Interleukin; CysLT: Cysteinyl-leukotrienes.

added GC. Also, bone-marrow from unsensitized C57BL/6 mice shows eosinophilia *in vivo* after dexamethasone administration[53], in the absence of any known CysLT inducer, just as dexamethasone enhances eosinopoiesis in culture without addition of CysLT. The effectiveness of both partners in the absence of the other is thus established, showing that they have independent pharmacological effects outside the framework of sensitization/challenge. Nevertheless, blocking either inside this framework achieves full prevention of the effects of challenge. This suggests that during challenge they become functionally coupled *in vivo*, which does not necessarily occur following dexamethasone administration. Coupling is here characterized by continuity in time (one event follows the other) and by dependence of the latter event on the former (blockade of the former event prevents the latter). However, it is not synonimous with causality in the usual sense: The first event might be just permissive for the second, not necessarily its immediate cause. Coupling is a term applicable to events that take place at separate moments in separate sites, just as it is to events that take place at separate moments in very close sites or even at exactly the same site, provided the second event is reproducibly prevented by blockade of the first. These distinct possibilities are illustrated by the two modes of cytokine action discussed in section 2.

Our hypothesis (coupling of GC and 5-LO in response to challenge) is consistent with the observed difference between the effects of challenge and of *in vivo* exposure to dexamethasone in the BALB/c strain. Challenge induces eosinophilia and primes for better responses to IL-5. Dexamethasone does not induce eosinophilia, but does good priming. Challenge effects are GC-dependent, with both eosinophilia and priming being abolished by RU486. Hence, even though GC are central to challenge, there is a hitherto unidentified factor present *in vivo* during challenge in addition to elevated GC, which modifies the ultimate effects of GC on bone-marrow, by coupling GC to CysLT. Below we develop the hypothesis that this unidentified factor is TNF-α, also produced in the course of challenge, and capable of inducing both GC[45] and CysLT[73,74].

Further insight can be provided by a comparison of dexamethasone and challenge: Dexamethasone-exposed eosinophils are cytologically immature and show no resemblance to circulating eosinophils[36,51]; challenge-induced eosinophils[22], as well as those induced by CysLT *in vitro*[70], are fully mature. In a sense, dexamethasone is an incomplete enhancer of eosinopoiesis, for it increases

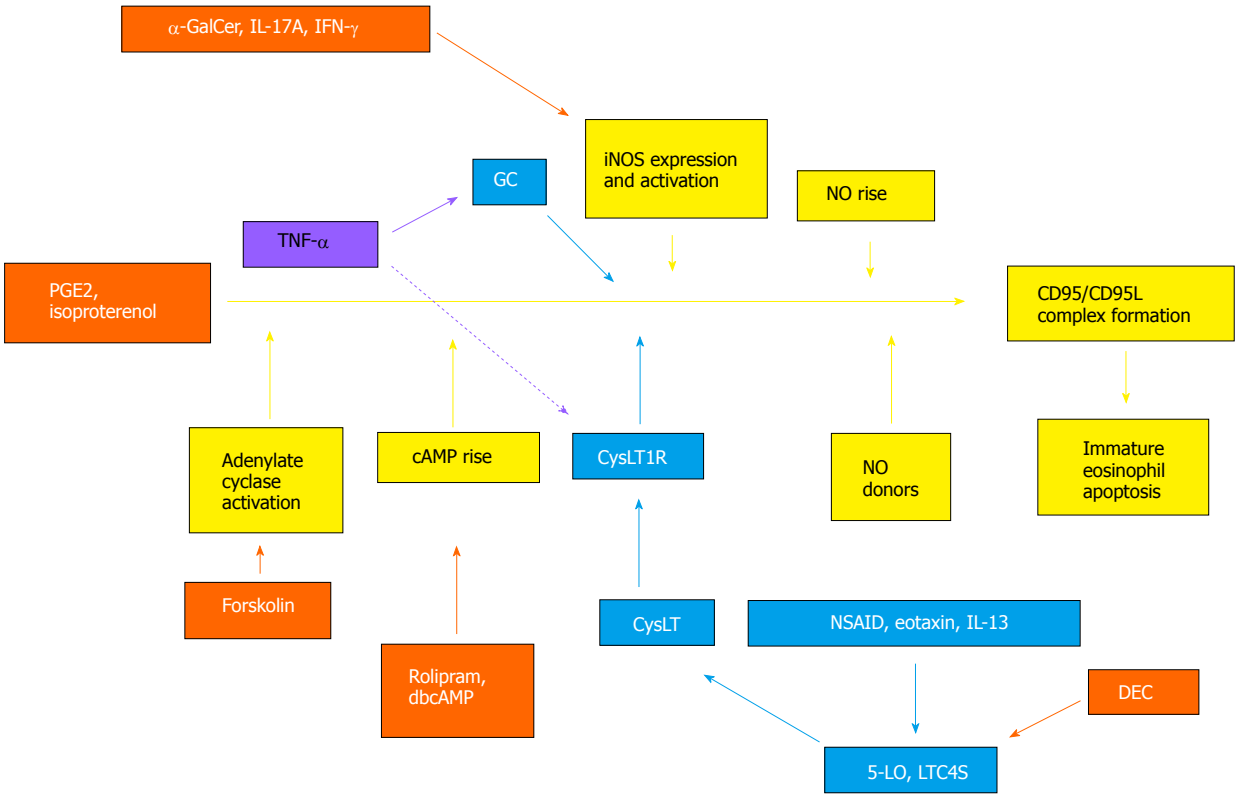

Figure 2 A graphical abstract of the main events identified in extrinsic regulation of bone-marrow eosinopoiesis, and of the hypothetical interactions of tumor necrosis factor-α with the underlying mechanisms. Colored boxes and arrows identify different classes of agents and their actions as follows: Orange, extrinsic suppressors of murine bone-marrow eosinopoiesis both *in vitro*[23,26,72,73] and *in vivo*[47,54]; light blue, extrinsic enhancers of eosinopoiesis *in vitro*[25,36,51,70] and *in vivo*[45,47,52,53]; yellow, essential components of a proapototic sequence (iNOS-CD95L-dependent pathway[54,72,73]) which is susceptible to activation by the first (orange-labeled) and blockade by the second (light blue-labeled) sets of extrinsic regulators; lavender, TNF-α, presenting both constitutive (continuous arrow) and challenge-induced (discontinous arrow) effects, inside the bone-marrow, besides its extramedullary actions[45] as activator of the HPA axis (not shown). TNF-α: Tumor necrosis factor-α.

the number of eosinophil-lineage cells but prevents their full maturation. The maturation sequence, which involves downregulation of α4 integrin in dexamethasone-exposed immature eosinophils, can be completed in the presence of PGE2[36] or IL-17[26] *in vitro*, just as it is completed in the presence of CysLT *in vivo*[47].

To reconcile all of these apparent paradoxes, we propose a model in flow chart format (Figure 1) which has the following essential tenets: (1) GC and CysLT become functionally coupled *in vivo* as a consequence of allergen challenge, so that TNF-α-dependent GC signaling makes it possible for CysLT generated locally in the context of allergic challenge to induce bone-marrow eosinophilia; (2) because the upstream permissive element (GC) and the downstream effector element (CysLT) are coupled, blocking the former will prevent the actions of the latter; (3) as a result of this coupling, full maturation of the eosinophils produced can be achieved *in vivo* after challenge; and (4) nevertheless, this mechanism is not operating unchecked *in vivo*, for its operation makes it less effective in subsequent rounds of allergen challenge, as shown by the attenuation of the proallergic effects of CysLT *ex vivo*, and by the observation that the positive effects of GC on the eosinophil lineage tend to less marked following chronic (repeated challenge) than acute (single challenge) exposures. Attenuation of CysLT effects *ex vivo* in murine bone-marrow would be consistent with observations that allergen challenge leads to a large increase in CysLT production in humans, and that a mechanism of desensitization to CysLT effects may limit the untoward effects of these potent bronchoconstrictors[75], although at present it is unclear whether and how this applies to hemopoietic effects.

In our model, we propose a role for TNF-α, not only in inducing GC production through HPA axis activation, but in coupling GC surge to CysLT production. The hypothesis of a complex relationship of TNF-α produced after challenge to actions of GC and CysLT is illustrated in Figure 2, which provides a graphical abstract of the essential biochemical events identified so far in the extrinsic regulation of bone-marrow eosinopoiesis by allergen challenge, drugs and cytokines. Coupling of GC to CysLT is portrayed, based on the available evidence, as a transient relationship between events taking place within a single cell target (eosinophil precursor inside the bone-marrow), since these events all impinge upon a signaling sequence that begins at surface receptors and ends at apoptotic cell death[72,73] which is limited to immature eosinophils and their immediate precursos[36]. In this continuous sequence of signaling events, GC and CysLT act as suppressors

of apoptosis (indicated in light blue colored boxes and arrows) by blocking distinct signaling steps upstream from iNOS; conversely, inhibitors of CysLT production or action, including DEC, promote apoptosis (indicated in orange-colored boxes and arrows, for DEC as well as a wide panel of pharmacological agents which act at CysLT-unrelated steps) by acting on targets upstream from iNOS[23,26,45,47,54,70,72,73]. In addition to its systemic effects on adrenal release of GC during allergen challenge, which are not shown in the picture, TNF-α is hypothesized to have separate effects on GC and CysLT-mediated responses: A constitutive effect permissive for GC action on eosinophil precursors (solid lavender line), and an adaptive (coupling) effect permissive for GC control of CysLT responses in the same cell target (discontinuous lavender line). TNF-α has been reported by others to induce CysLT production and the expression of critical enzymes in CysLT biosynthesis; in addition, LTD4 duplicates these effects, providing a mechanism for amplification of TNF-α actions[74]. It at present is unclear whether these observations from other groups apply to bone-marrow, and, if so, how TNF-α induction of CysLT might be dependent on GC signaling.

To test the validity of these models, some points are critical, foremost the definition of the site, timing and mechanism of coupling of GC to CysLT, and of the role played by TNF-α therein. To define the mechanisms of attenuation of CysLT-dependent response is equally essential, including the roles played by GC hormones themselves and by changes in CysLT receptor types, expression or intracellular signaling. These steps should help us put in proper perspective the paradoxical enhancement of eosinophil production by GC, which, despite being at odds with the prevailing views of the contributions of GC and eosinophils to immune responses, is likely to shed some light on the puzzle of stress-related mechanisms in allergic disease.

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ORIGINAL ARTICLE

Retrospective Study

Statin escape phenomenon: Fact or fiction?

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Abstract

AIM

To evaluate the presence of the so called "statin escape" phenomenon among hyperlipidemic subjects attending a lipid clinic.

METHODS

This was a retrospective analysis of 1240 hyperlipidemic individuals followed-up for ⩾ 3 years. We excluded those individuals meeting one of the following criteria: Use of statin therapy at baseline visit, discontinuation of statin treatment at most recent visit, change in statin treatment during follow-up and poor compliance to treatment. Statin escape phenomenon was defined as an increase in low-density lipoprotein cholesterol (LDL-C) levels at the most recent visit by > 10% compared with the value at 6 mo following initiation of statin treatment.

RESULTS

Of 181 eligible subjects, 31% exhibited the statin escape phenomenon. No major differences regarding baseline characteristics were found between statin escapers and non-statin escapers. Both escapes and non-escapes had similar baseline LDL-C levels [174 (152-189) and 177 (152-205) mg/dL, respectively]. In comparison with non-escapers, statin escapers demonstrated lower LDL-C levels at 6 mo after treatment initiation [88 (78-97) mg/dL *vs* 109 (91-129) mg/dL, $P < 0.05$], but higher levels at the most recent visit [103 (96-118) mg/dL *vs* 94 (79-114) mg/dL, $P < 0.05$].

CONCLUSION

These data confirm the existence of an escape phenomenon among statin-treated individuals. The clinical significance of this phenomenon remains uncertain.

Key words: Statin; Escape; Cholesterol

Core tip: This was a retrospective study aiming to evaluate the presence of the so called "statin escape" phenomenon among hyperlipidemic subjects attending a lipid clinic and elucidate any potential confounding factors. This study confirms the limited bibliography reporting on statin escape phenomenon and its quite high prevalence. However, due to the small number of eligible participants, we were not able to identify potential predictors for the statin-escape phenomenon or establish an association between statin escape and incidence of cardiovascular disease. In this context, further investigation on the underlying pathophysiology of this phenomenon and its potential clinical ramifications is required.

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INTRODUCTION

Statins remain the cornerstone therapy for primary and secondary cardiovascular prevention, mainly due to their ability to reduce low-density lipoprotein cholesterol (LDL-C)[1]. Nevertheless, a notable cardiovascular risk remains in statin-treated individuals, which has been attributed to other residual factors, such as hypertension, diet and adherence to therapy[2]. Recently, the so called "statin escape" phenomenon has been reported as an independent cardiovascular risk factor in patients with acute myocardial infarction on prolonged statin treatment[3]. This phenomenon was first described in small studies including patients with familial hypercholesterolemia[4,5] and afterwards in the Expanded Clinical Evaluation of Lovastatin (EXCEL) study[6]. The latter reported an increase in LDL-C levels after the first year of statin treatment, despite a marked decrease in those levels 1 mo after treatment initiation[6]. So far there have been few reports on this phenomenon and its underlying mechanisms remain obscure[5,7-9].

The aim of this study was to provide additional data on the possible statin escape phenomenon based on the experience of a lipid clinic and try to elucidate potential risk factors.

MATERIALS AND METHODS

This was a retrospective (from 1999 to 2013) observational study as previously described[10-12]. Briefly, dyslipidemic adults followed-up for ⩾ 3 years in the Outpatient Lipid Clinic of the University Hospital of Ioannina in Greece were included. A complete assessment of serum lipid profile along with cardiovascular risk factors and concomitant treatment was available. The study protocol was approved by the Institutional Ethics Committee.

Demographic characteristics as well as various clinical and laboratory data were recorded at the baseline visit, at 6 mo and the most recent visit. These included: (1) age, gender, and smoking status; (2) body mass index (BMI) and waist circumference; (3) fasting glucose levels and glycated hemoglobin (HbA1c); (4) blood pressure (BP); (5) estimated glomerular filtration rated (MDRD - eGFR); and (6) a complete fasting lipid profile, including total cholesterol (TCHOL), triglycerides (TGs), high-density lipoprotein cholesterol (HDL-C), LDL-C and non-high density lipoprotein cholesterol (non-HDL-C). The methods of blood sample collection and biochemical assessments have been previously described[10].

The evaluation of adherence to medication was based on the Hellenic national e-prescription web database. Subjects were classified according to their compliance with treatment as good or poor compliers if they refill ⩾ or < 80% of their expected prescriptions over time, respectively. We excluded those individuals meeting one of the following criteria: Use of statin therapy at baseline visit, discontinuation of statin treatment at most recent visit, change in statin treatment during follow-up and poor compliance to treatment. Statin escape phenomenon was defined as an increase in subject LDL-C levels at the most recent visit by > 10% compared with the value at 6 mo following initiation of statin therapy[8].

Statistical analysis

Continuous variables were tested for normality by the Kolmogorov-Smimov test and logarithmic transformations were performed if necessary. Data are presented as mean ± standard deviation (SD) and median [interquartile range (IQR)] for normal and non-normal distributed data, respectively. χ^2 tests were performed for categorical values. The difference of variables between ⩾ 2 groups was assessed by analysis of variance (ANOVA) and *post hoc* least significant difference tests were used for the comparison of variables or ratios of interest between the groups. Paired sample *t* tests were performed to assess the change of variables within each study group. Analysis of covariance (ANCOVA) was performed to assess the difference of variables between 2 subject groups, after adjusting for their baseline values. Binary logistic regression was performed to elucidate potential predictors for statin escape phenomenon. Two tailed significance was defined as $P < 0.05$. Analyses were performed with the Statistical Package for Social Sciences (SPSS), v21.0 software (SPSS IBM Corporation, Armonk, New York, United States).

RESULTS

Of 1240 hyperlipidemic individuals, 181 were considered eligible for the present analysis (Figure 1). Study participant baseline characteristics are shown in Table 1. Of 181 eligible subjects, 56 (31%) exhibited the statin escape

Table 1 Baseline characteristics of study participants

Variable	Escape group	Non-escape group
n	56	125
Gender (male), %	43	52
Current smoking, %	9	14
Age, yr	56 (51-63)	57 (49-65)
Waist, cm	97 (90-101)	98 (90-105)
SBP, mmHg	134 (127-146)	140 (129-150)
DBP, mmHg	83 (79-95)	87 (80-92)
Follow-up, yr	4 (3-6)	4 (4-7)
Metabolic syndrome, %	39	40
Hypertension, %	59	57
Diabetes, %	11	9
Stroke, %	5	4
Coronary heart disease, %	7	1[a]
Abdominal aortic aneurysm, %	2	0
Carotid stenosis ⩾ 50%, %	0	2
Peripheral arterial disease, %	0	1
Statin therapy and median dose, % (median dose)		
Atorvastatin	38 (20 mg)	34 (20 mg)
Rosuvastatin	29 (10 mg)	24 (10 mg)
Simvastatin	21 (40 mg)	26 (40 mg)
Fluvastatin	7 (80 mg)	6 (80 mg)
Pravastatin	0	1 (40 mg)
β-blocker, %	9	7
Thiazides, %	11	19
Pioglitazone, %	4	1
Antipsychotics, %	0	1
Levothyroxine, %	4	5
Clopidogrel, %	2	2
Proton-pump inhibitors, %	4	4

Median follow-up duration = 4 years (IQR: 3-6 years). Values are expressed as median (IQR), unless percentages as shown. [a]$P < 0.05$ for the comparison with the escape group. DBP: Diastolic blood pressure; IQR: Interquartile range; SBP: Systolic blood pressure.

phenomenon and 125 (69%) did not. There were no differences between these 2 groups apart from the higher baseline prevalence of coronary heart disease noticed in the escape group (7% *vs* 1%, $P < 0.05$). As shown in Table 1, there was no difference between the 2 groups regarding statin treatment. No participant received any non-statin lipid-lowering therapy (*i.e.*, fibrate, ezetimibe). In addition, no difference was found regarding drugs interfering with cholesterol or statin metabolism (*i.e.*, β-blockers, thiazides, pioglitazone, atypical antipsychotics, levothyroxine, clopidogrel or proton-pump inhibitors; Table 1).

Baseline lipid and metabolic profile did not differ between the 2 study groups (Table 2). Six months after the initiation of statin treatment, LDL-C levels were lower in the escape compared with the non-escape group [88 (78-97) mg/dL *vs* 109 (91-129) mg/dL, $P < 0.01$; Figure 2]. On the contrary, LDL-C levels at the most recent visit were lower in the non-escape compared with the escape group [103 (96-118) mg/dL *vs* 94 (79-114) mg/dL, $P < 0.01$; Figure 2]. Similarly, non-HDL-C levels were lower six months after the initiation of statin therapy in the escape compared with the non-escape group among non-diabetic individuals [107 (97-121) mg/dL *vs* 132 (115-153) mg/dL, $P < 0.01$; Table 2]. On the other hand, higher non-HDL-C levels were noticed in the former group at the most recent visit (Table 2). TRG significantly declined by 11% and 18% in the escape and non-escape group during follow-up, respectively ($P < 0.01$ respectively for the change within each group; Table 2). Despite the fact, that the non-escape group exhibited higher TRG levels than the escape group 6 mo after the initiation of statin therapy [104 (83-140) mg/dL *vs* 97 (69-117) mg/dL, $P < 0.05$], there was no difference between 2 groups regarding TRG levels at the most recent visit and the change of TRG levels during follow-up (P = NS for the comparison between 2 groups). On the other hand, HDL-C levels did not change during follow-up and were not different between 2 groups (Table 2).

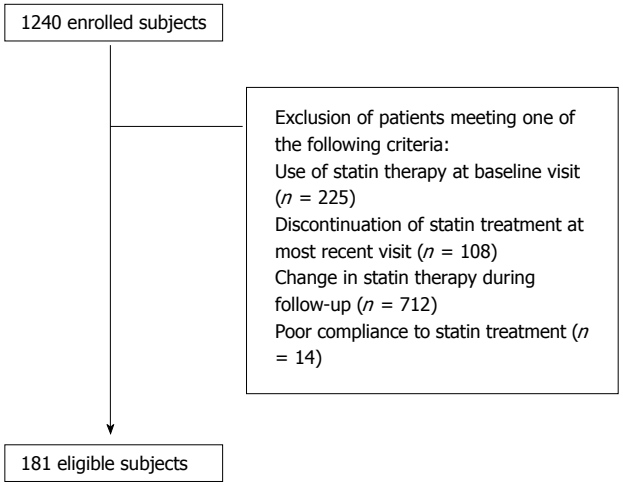

Figure 1 Flow chart of subject eligibility.

There was no significant difference between the 2 groups regarding BMI change. As also shown in Table 2, glucose levels did not change during follow-up and were not different between the 2 groups. eGFR declined by 0.5 and 4.1 mL/min per 1.73 m^2 in the escape and non-escape group, respectively ($P < 0.05$ respectively for the change within each group), but the difference between the 2 groups was not significant. The same was true for the change in diabetics' HbA1c levels (Table 2, P = NS for the comparison between the 2 groups).

Binary logistic regression assessing baseline characteristics along with the changes in BMI, eGFR or HbA1c levels during follow-up did not reveal any significant predictor for the statin escape phenomenon.

During a median follow-up of 4 years, 1 of 56 escape individuals and 6 of 125 non-escape subjects were diagnosed with incident cardiovascular disease (P = NS for the comparison between the 2 groups).

DISCUSSION

The present report confirms the existence of statin escape phenomenon in clinical practice.

Two small studies including patients with familial hypercholesterolemia were the first to notice a paradox rebound cholesterol increase following statin dose

Table 2 Lipid and metabolic profile of study participants

	Baseline visit	Visit at 6 mo	Most recent visit
TCHOL, mg/dL			
Escape group	258 (233-283)	162 (147-174)	182 (170-201)
Non-escape group	259 (235-295)	184 (162-206)[a]	172 (154-193)[a]
TG, mg/dL			
Escape group	117 (89-175)	97 (69-117)	104 (87-129)
Non-escape group	132 (99-181)	104 (83-140)[a]	108 (79-130)
HDL-C, mg/dL			
Escape group	53 (47-68)	55 (43-64)	54 (48-68)
Non-escape group	53 (46-65)	52 (44-60)	56 (46-62)
LDL-C, mg/dL			
Escape group	174 (152-189)	88 (78-97)	103 (96-118)
Non-escape group	177 (152-205)	109 (91-129)[a]	94 (79-114)[a]
Non-HDL, mg/dL[1]			
Escape group	204 (181-223)	107 (97-121)	127 (116-143)
Non-escape group	209 (182-241)	132 (115-153)[a]	118 (102-137)[a]
BMI, kg/m^2			
Escape group	27.3 (23.5-29.9)	27.2 (23.5-30.1)	27.6 (24-30.2)
Non-escape group	27.9 (25.5-30.6)	28.3 (25.1-30.9)	28.4 (25.5-31.5)
Fasting glucose, mg/dL			
Escape group	95 (88-105)	95 (87-129)	95 (88-106)
Non-escape group	93 (87-103)	94 (88-104)	96 (89-106)
HbA1c, %[2]			
Escape group	8.5 (6.7-8.6)	6.6 (5.6-5.9)	6.7 (6.6-7.1)
Non-escape group	8.4 (7.7-10.9)	6.7 (6.3-7.9)	6.9 (6.3-7.6)
MDRD-eGFR, mL/min per 1.73 m^2			
Escape group	77 (69.6-86.7)	76.6 (67.9-84.8)	76.5 (65.4-81)
Non-escape group	81 (70.7-91.4)	79.7 (69-89.7)	76.9 (65.5-85.7)

Values are expressed as median (IQR). To convert from mg/dL to mmol/L multiply by 0.0555 for glucose, 0.02586 for TC, HDL-C, LDL-C, and 0.01129 for TG. [1]Non-HDL-C levels refer to non-diabetic individuals (n = 164); [2]HbA1c values refer to diabetic individuals (n = 17). [a]P < 0.05 for the comparison with the escape group. BMI: Body mass index; MDRD-eGFR: Estimated glomerular filtration rate according to The Modification of Diet in Renal Disease (MDRD) Study equation; HbA1c: Glycated hemoglobin; HDL-C: High-density lipoprotein cholesterol; IQR: Interquartile range; LDL-C: Low-density lipoprotein cholesterol; non-HDL-C: Non-high-density lipoprotein cholesterol; TCHOL: Total cholesterol; TG: Triglycerides.

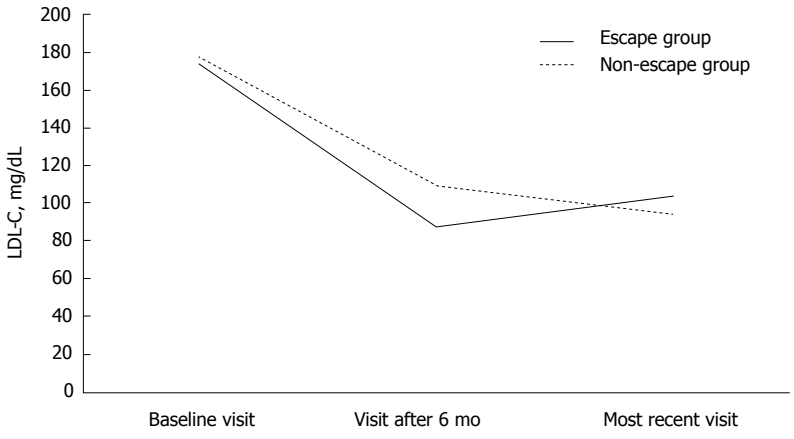

Figure 2 Change in low-density lipoprotein cholesterol levels during follow-up. [a]P < 0.05 for the comparison between the 2 groups. LDL-C: Low-density lipoprotein cholesterol.

increase[4,5]. Since then, the EXCEL study along with others, has described this so called statin escape phenomenon[3,5-7]. Our results showing an initial marked LDL-C reduction but followed by a > 10% LDL-C increase after prolonged statin treatment in subjects exhibiting the statin escape phenomenon are in line with the results of these studies[3,5,7]. Similar to previous studies, we did not find any predictors for this phenomenon[3,5,7]. A recent study showed that statin escape phenomenon not only exists, but also might be an independent predictor of cardiovascular disease[3]. The mechanisms attributing to the statin escape phenomenon have not yet been elucidated. The failure of statin therapy to decrease LDL-C levels on a long-term basis may be attributed to poor compliance with lipid-lowering treatment and diet. Particularly, an increased intake of cholesterol in the diet may contribute to intermittent variations in cholesterol levels. In addition, weight changes or a poor glycemic

control in diabetic individuals could also cause a LDL-C increase, which could be wrongfully considered as statin escape phenomenon. After excluding subjects with these characteristics, one study concluded that only 1.2% of 161 study participants exhibited the statin escape phenomenon, although 28% of those were initially considered to meet the criteria of statin escape[7]. Despite the fact that no data regarding diet and exercise was available in our study, there was no significant difference between groups in terms of BMI change, glycemic control and kidney function.

We also assessed non-HDL-C levels in non-diabetic individuals considering that atherogenic dyslipidemia may alter LDL-C changes[10]. Statin escapers had higher non-HDL-C levels after prolonged statin therapy in comparison with non-escapers, although they had a higher non-HDL-C reduction 6 mo after treatment onset.

Although we checked for adherence to therapy, our study might have included non-compliant individuals. It may be possible that the escapers adhered less to statin therapy and diet after seeing a large drop in their LDL-C levels. Another possible explanation for the statin escape phenomenon could be the concomitant therapy, since a variety of drugs could increase LDL-C lowering action of statins by inducting cytochromes CYP450-3A4 and 2C9[13,14]. According to a few experimental studies, statin escape phenomenon could be attributed to a slow increase in the 5-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity or to an increase in proprotein convertase subtilisin kexin-like 9 (PCSK9) levels caused by prolonged statin therapy[9,15-19].

Our data suggest that statin escape phenomenon is indeed noticed in clinical practice, although its clinical significance remains uncertain. Patients with larger than anticipated initial LDL-C lowering should be carefully monitored.

COMMENTS

Background

A few studies have reported on the so called "statin escape phenomenon", which describes an increase in low-density lipoprotein cholesterol (LDL-C) levels after prolonged statin therapy despite an initial marked decrease. Statin escape phenomenon has been recently reported as an independent cardiovascular risk factor.

Research frontiers

Very few studies have reported on statin escape phenomenon and its underlying mechanisms remain obscure. The present study contributes to clarifying whether this phenomenon exists in clinical practice.

Innovations and breakthroughs

This was a retrospective observational study with a small sample size. However, only the EXCEL study, which is the only randomized trial reporting on statin escape phenomenon and a retrospective cohort had larger samples. The small number of eligible participants did not allow any analysis to identify potential predictors for the statin-escape phenomenon. Additionally, due to small sample and low incidence of cardiovascular disease this study did not have the power to stablish an association between statin escape and incidence of cardiovascular disease. Nevertheless, this study confirms the limited bibliography reporting on statin escape phenomenon and its quite high prevalence (28%-31%).

Applications

This study suggests that further investigation on the underlying pathophysiology of the statin escape phenomenon and its potential clinical ramifications is required.

Peer-review

This study is well written and the patients have been well selected although several variables could have influenced the results.

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ORIGINAL ARTICLE

Observational Study

Discernment scheme for paraquat poisoning: A five-year experience in Shiraz, Iran

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Abstract

AIM

To evaluate various schemes for paraquat poisoning and different variables that influence the outcome of acute paraquat poisoning.

METHODS

In a cross-sectional study, the information about all cases of acute paraquat poisoning who were admitted to teaching hospitals affiliated to Shiraz University of Medical Sciences, in a five year period (September 2010 to September 2015) were evaluated. The variables included: Demographic data, medical assessment, therapeutic options, laboratory findings, and the outcomes. Data were

analyzed using SPSS, version 22. Significant difference between groups was tested using t-test for continues outcomes and χ^2 test for categorical. The significance level was considered to be $P < 0.05$.

RESULTS

A total of 104 patients (66.3% male) were evaluated. The mean age of the female patients was 22.81 ± 9.87 years and the male patients' was 27.21 ± 11.06 years. Ninety seven (93.3%) of all the cases were suicide attempts with mortality rate of 43.2%. Despite the necessity for emergency hemodialysis during the first 6 h of intoxication, none of the patients had dialysis during this time. Immunosuppressive and corticosteroid medications were not administrated in adequate dosage in 31.1% and 60% of the patients, respectively. Ingestion of more than 22.5 cc of paraquat and increase in creatinine level were the most important predictors of mortality.

CONCLUSION

Treatment should start immediately for these patients. Moreover, creating a clinical guideline according to the findings can have an impact on the treatment procedure which seems to be necessary.

Key words: Mortality; Paraquat; Poisoning; Prognosis; Suicide

Core tip: In developing countries with an agriculture economy poisoning by means of herbicides is very common. Paraquat is a highly toxic compound and consumption of 30 mg/kg is lethal in humans. In this study, we have analyzed multi-center data of patients with paraquat poisoning between September 2010 and September 2015, establishing the largest series of paraquat poisoning in the Middle East. Based on the data, medical knowhow that affects its current management as well as different variables which influence the outcome were evaluated.

Kavousi-Gharbi S, Jalli R, Rasekhi-Kazerouni A, Habibagahi Z, Marashi SM. Discernment scheme for paraquat poisoning: A five-year experience in Shiraz, Iran. *World J Exp Med* 2017; 7(1): 31-39 Available from: URL: http://www.wjgnet.com/2220-315X/full/v7/i1/31.htm DOI: http://dx.doi.org/10.5493/wjem.v7.i1.31

INTRODUCTION

Due to widespread usage of herbicides in agricultural industry reports of human poisoning has been on the rise around the world[1-3]. Paraquat (1,1′-dimethyl-4,4′ bipyridinium dichloride) is a well-known compound used in agriculture and it is a suitable due to its wide range of effects on weeds and instability in the environment[4,5].

Consumption of 30 mg/kg (equal to around 3-6 g of paraquat ion) is lethal in adults[6-8]. In the case of oral intake, it is quickly absorbed through the luminal tract, and 95% of its tissue distribution occurs within the first 6 h. Kidneys play a vital role in disposing paraquat from the body, and its maximum disposal is carried out during the first 12 to 24 h[6]. Symptoms include: Burning sensation in the mouth, throat, chest, epigaster, nausea, vomiting, abdominal pain, and diarrhea, which can be stopped after 2-3 d, if the patient is still alive[9]. Additionally if a patient has consumed more than 20 mg/kg of paraquat ion, his/her survival rate is very low[10]. I think (equal to 10 cc of 20% solution) is not a proper statement, because 20 mg/kg is more explanatory itself. The main mechanism in being poisoned with paraquat is the formation of superoxide ions, active oxygen radicals, NADPH oxidation, lipid peroxidation of cell membrane, and destruction of the cell membrane structure[4,11]. Despite progressions in critical care domain, to this point there has not been any effective treatment for paraquat poisoning. Some studies have indicated some improvement in prognosis of patients, ensuing the prescription of absorbents, treatment by immunosuppressive medications, radiotherapy and hemodialysis[12-14]. The main objective in this research was to study the clinical symptoms, laboratory abnormalities and the outcome of paraquat poisoning in a 5-year period in Fars province, Iran.

MATERIALS AND METHODS

Study population and data collection

In this retrospective descriptive analytical study, a total of 104 records of paraquat poisoning patients in three main tertiary hospitals in Shiraz, Iran, from September 2010 to September 2015 were evaluated. This research was conducted following the approval of Shiraz University of Sciences Ethics Committee. The required data were manually obtained from the patients' records. The data included; age, gender, consumed paraquat amount, occurrence of vomiting after consumption, the gap between poison consumption and treatment initiation, the treatments modalities, hospitalization duration, laboratory abnormalities, and sequela. Patients who had not signed the written informed consent for using their records, those who had taken other medications or poisons simultaneously, and those who had a history of cardiac, pulmonary, renal or hepatic diseases were excluded from the study.

Statistical analysis

The retrieved data were analyzed using SPSS, version 22. The continuous variables were described by standard deviation ± mean and the categorical were reported in the form of frequencies. Significant difference between groups was tested using *t*-test for continues outcomes and χ^2 test for categorical. In all cases, the significance level was considered to be $P < 0.05$. In order to assess the performance of laboratory changes in predicting the death occurrence, the area under receiver operating characteristic (ROC) curve and sensitivity, specificity, positive and negative predictive value were studied. The

Table 1 Baseline demographics of the subjects, 2010-2015

Variable	Total = 104
Gender	
Male (%)	69 (66.3)
Female (%)	35 (33.7)
Duration of hospitalization	
1-6 d (%)	61 (58.7)
7-13 d (%)	29 (27.9)
More than 14 d (%)	14 (13.5)
Mean (d)	6.73 ± 5.73
Time interval (d)	1-27
Cause of poisoning	
Occupational exposure (%)	4 (3.8)
Suicidal (%)	97 (93.3)
Accidental (%)	3 (2.9)
Habitat	
Rural (%)	80 (76.9)
Urban (%)	24 (23.1)
Type of poisoning	
Ingestion (%)	101 (97.1)
Injection (%)	3 (2.9)
Outcome	
Recovery (%)	59 (56.7)
Death due to complications (%)	45 (43.3)

confidence interval was 95%.

RESULTS

Overall characteristics of patients

In this research, a total of 104 patients poisoned by consuming paraquat were studied in a period of five years. The demographic data of the patients are presented in Table 1.

The duration of hospital stay for the patients was 6.73 ± 5.73 d (between 1 and 27 d) on average. Poisoning with paraquat in males was 1.9 times higher than females. The highest rate of poisoning prevalence was among females under 20 and males between the ages of 20 and 30. The mean age of the female patients was 22.81 ± 9.87 (between 1 and 61 years) and the male patients' was 27.21 ± 11.06 (between 15 and 60 years) (P = 0.045).

Clinical manifestations at presentation

Majority of the patients (76 cases; 73.1%) had vomited before being admitted to the hospital and the most common symptom during admission was nausea (74 cases; 71.1%). However, prevalence of epigasteric pain and inflammation of the oral mucosa was (29 cases; 27.9%) and (28 cases; 26.9%). No dysrhythmia was observed on the electrocardiogram at the time of presentation or during hospitalization, excluding agonal arrhythmia in dying patients.

Emergency management of poisoned patients

The most common decontamination method carried out for the patients was gastric lavage in 94 cases (90.4%). Charcoal alone or along with Fuller's earth was prescribed for gastric decontamination in 60 (57.7%) and 17 cases (16.3%), respectively. Gastric lavage was carried out in all cases that Fuller's earth or charcoal was prescribed. Only in 17 cases, gastric lavage was the sole method carried out.

Medical knowhow and inappropriate treatments

In 91 cases (87.5%), treatment was carried out by corticosteroids and in 39 (37.5%) by cyclophosphamide. In 50 cases (48.1%) N-acetyl cysteine (NAC), in 34 (32.7%) vitamin E, and in 32 (30.8%) vitamin C were prescribed as antioxidant medications.

In none of the 45 deceased patients, treatments were carried out completely. The most common type of managements were prescribing corticosteroid medications in 43 cases (95.6%), gastric lavage in 42 (93.3%), charcoal in 35 (77.78%), and NAC administration in 22 (48.9%) patients. Lack of attention in prescribing cyclophosphamide, dexamethasone and vitamin E as the most commonly ignored treatments in deceased patients, had occurred in 28 cases (62.2%), and lack of attention in prescribing NAC had occurred in 23 cases (51.1%). Methylprednisolone was not prescribed in 9 cases (20%), and in 27 cases (60% of the deceased patients), it was prescribed insufficiently.

Since, hemoperfusion was not available in any of the tertiary hospitals in Shiraz, Iran therefore; hemodialysis was carried out for extracorporeal removal of paraquat. Only 3 cases had expired due to the severity of poisoning during the early hours of admission and before performing hemodialysis. Nonetheless, initiating hemodialysis was delayed in all cases, at least for 6 h, mainly due to the delay in receiving the results of viral marker status. About 54% of the patients were hemodialyzed again due to increase in renal biomarkers after the first day.

Chest radiographic findings

Lung radiography was done for 45 cases. Table 2 presents the frequency of the positive lung radiography findings in the studied patients.

Correlation between the amount of consumed poison and prognosis

The amount of consumed poison in patients was 34.61 ± 55.36 mL (between 1.5 and 300 mL) on average. While the deceased patients had consumed 66.63 ± 72.61 mL of poison on average, this amount was 10.18 ± 5.77 mL on average for the patients who survived (P = 0.001); that is, 85.5% of the patients who had consumed less than 10 cc and 12.7% of the patients who had consumed between 10 and 20 cc of poison were discharged after recovery. On the other hand, 91.1% of the patients who had consumed more than 20 cc of poison ultimately expired (P = 0.000).

Figure 1, illustrates the ROC curve related to poison consumption and mortality rate in patients. Table 3 shows the cutoff point for the amount of poison consumed and patients' mortality, by considering the minimum positive predictive value of 90%. Based on poison consumption

Table 2 Chest radiographic findings of the 45 survivors and non-survivors among paraquat poisoned patients

Radiographic findings	Time interval of radiographic study (d)	No. of patients (percent in the survivors/non-survivors groups)
Non-survivors (n = 18)		
Pneumothorax (%)	2-5	2 (11.1)
Pneumomediastinum/emphysema (%)	1-2	2 (11.1)
ARDS (%)	1-10	4 (22.2)
Lung fibrosis (%)	4-14	10 (55.6)
Survivors (n = 27)		
Pneumothorax (%)	1-5	2 (7.4)
Lung fibrosis (%)	4-32	4 (14.8)
Normal (%)	1-8	21 (77.8)

ARDS: Acute respiratory distress syndrome.

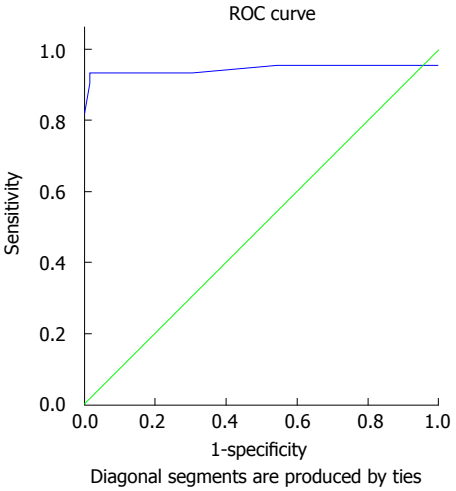

Figure 1 Receiver operating characteristic curve related to the position amount consumption in relation with patients' death. Based on the area under the curve, the confidence level was determined to be 95% for the poison consumption of 0.945 [between 0.87-1.00 (P = 0.000)]. ROC: Receiver operating characteristic.

rate and ROC curve, the best cutoff point was calculated at 22.5 cc or higher (considering the minimum positive predictive value of 90%).

On average patients' were deceased after 4.8 ± 4.62 d of hospitalization, (between the first and 21st days). Total of 9 cases expired during the first day of hospitalization who had consumed about 35 and 300 cc of poison.

Correlation between laboratory abnormalities and prognosis

This study indicates that maximum average levels of serum creatinine was 2.50 ± 1.80 (between 0.6 and 9.5), maximum average of blood urea nitrogen 16.42 ± 29.18 (between 6 and 66), maximum average of AST levels 114.52 ± 246.85 (between 8 and 1509), and maximum average of ALT 301.43 ± 145.31 (between 8 and 1803) were observed after the third day of admission.

Figure 2, illustrates the ROC curve related to Levels of serum creatinine, blood urea nitrogen (BUN), aspartate aminotransferase (AST), and alanine transaminase (ALT) after the third day of admission and mortality rate in patients. Table 4 shows the cutoff point of serum creatinine level, BUN, AST, and ALT on the third day in relation with patients' mortality by considering the minimum positive predictive value of 90%. The best cutoff point (considering the minimum positive predictive value of 90%) was calculated based on serum creatinine level, BUN, AST, and ALT on the third day and ROC curve. This cutoff point was calculated to be 1.95 or higher for creatinine level, 25 or higher for BUN level, 24.5 or higher for AST level, and 12 or higher for ALT level on the third day.

DISCUSSION

Being poisoned with herbicides in developing countries of South, East and Southeast Asia with an agriculture economy is very common[15]. In this study, a total of 104 paraquat poisoning cases in Fars province, which is one of the agriculture hubs in Iran, were studied in a 5-year interval. More than 65% of all the cases were male. The gender ratio of the paraquat poisoning in other studies was reported to be between 55% and 70% in males[5,6,10]. However, the mean age of female patients which was around 23 years, was 4 years lower than the male patients; this was in accordance with Kim's study[16]. The highest prevalence was observed in teenage girls and males between the ages of 20 and 30, which are considered as the active population. Around 77% of the patients were from rural areas. Other studies, also showed that poisoning was more common among the rural population, from 56% to 73%[6,13]. Around 93% of the cases were suicide attempts, which is in accordance with the results from other studies[5,6,13].

The most common clinical symptoms in patients included nausea, epigasteric pain and inflammation of the oral mucosa, which were in line with results from Cherukuri *et al*[9]. However, Sandhu *et al*[12] had reported that all patients diagnosed with paraquat poisoning experienced nausea and vomiting, but oral mucosal ulcers were reported in only 59% of the patients.

Gastric lavage was carried out as the most common type of emergency procedure in about 90% of all the cases, prescribing charcoal in about 58% and prescribing

Table 3 The cutoff point for the amount of poison consumption and patients' death

Variable	Area under the ROC (the minimum positive predictive value of 90%)	Cutoff point	Positive predictive value	Negative predictive value	Sensitivity	Specificity
Amount of consumed poison	0.945 (0.87-1.0)	22.5	93.3	98.3	93.3	98.3

ROC: Receiver operating characteristic.

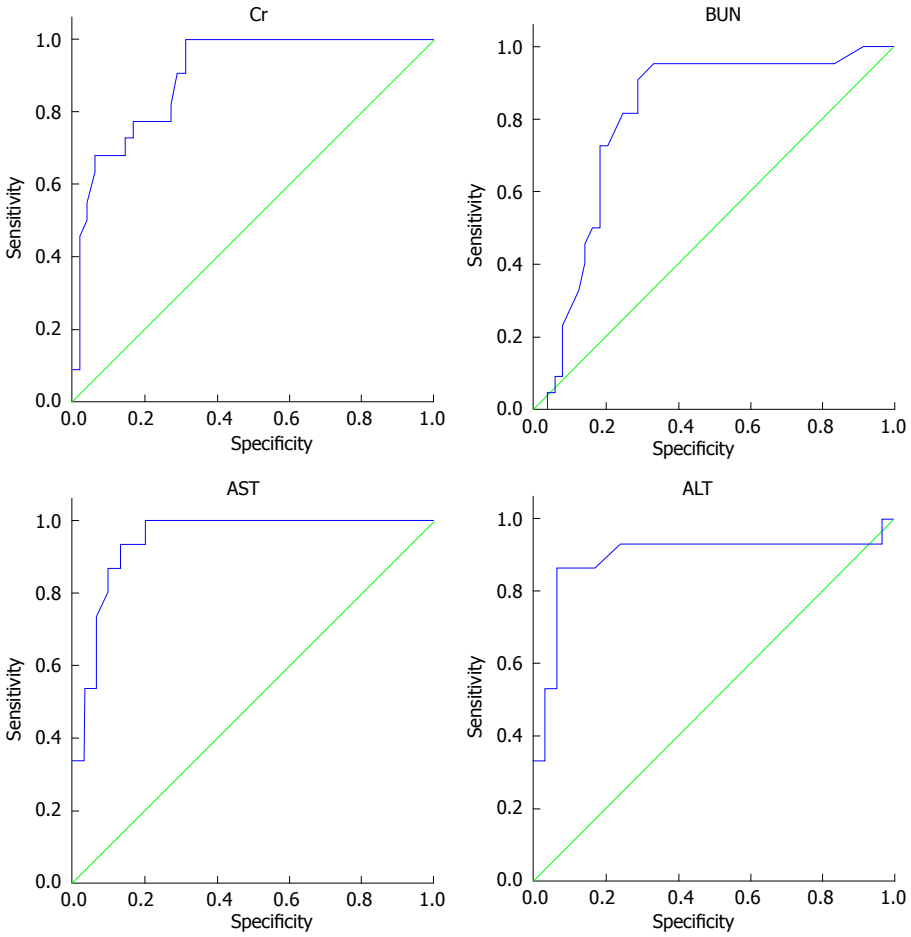

Figure 2 Receiver operating characteristic curve related to levels of serum creatinine, blood urea nitrogen, aspartate aminotransferase and alanine transaminase on the third day of hospitalization in relation with patients' death. Based on the levels under the curve, confidence interval of 95% was determined for creatinine on the third day 0.90 [between 0.83-0.97 (P = 0.000)], for BUN on the third day 0.80 [between 0.69-0.91 (P = 0.000)], for AST on the third day 0.85 [between 0.58-1.00 (P = 0.008)], and for ALT on the third day 0.79 [between 0.57-1.00 (P = 0.028)]. BUN: Blood urea nitrogen; AST: Aspartate aminotransferase; ALT: Alanine transaminase.

charcoal along with Fuller's earth was carried out in 16% of the all the patients in the present study. In Senarathna's study[17], it was shown that Fuller's earth was prescribed in about 75%, and charcoal prescription was carried out in about 22% of the cases; however, in 16% of their patients, both were prescribed. Nevertheless, it is worth mentioning that Fuller's earth (magnesium citrate) and charcoal have similar effects[18], and there is no need for their simultaneous prescription. Although some of the old studies had proposed the necessity for gastric lavage in paraquat poisoning[19,20], Wilks *et al*[21] showed that gastric lavage can lead to increase in mortality rate in cases where the patient has consumed lesser than 30 cc of poison. It seems that gastric cleansing was inappropriate in the studied patients.

Research has suggested that paraquat can reach plasma concentration peak in one hour due to rapid absorption[22,23], and subsequently accumulate in targeted tissues. However, there is a chance of re-distribution from tissues to plasma, as well. Paraquat distribution half-life is around five hours in human, and around 6 h after its consumption, it reaches the maximum of tissue concentration in the lungs[24,25]. Considering the pathology of free radicals in paraquat poisoning, some older studies have proposed the use of antioxidant medications such as vitamin E and vitamin C in order to reduce tissue injuries. However, the impact of such treatments has not been proven[26,27]. Also, NAC, as a proper source of

Table 4 Cutoff point for the levels of serum creatinine, blood urea nitrogen, aspartate aminotransferase and alanine transaminase on the third day in relation with patients' death considering the minimum positive predictive value of 90%

Laboratory variables in the third day of admission	Area under the ROC (the minimum positive predictive value of 90%)	Cutoff point	Positive predictive value	Negative predictive value	Sensitivity	Specificity
Serum creatinine	0.90 (0.83-0.97)	1.95	90.9	70.8	90.9	70.8
BUN	0.80 (0.69-0.91)	25	95.4	66.7	95.5	66.7
AST	0.85 (0.58-1.000)	24.5	100	66.78	100	72.7
ALT	0.79 (0.57-1.000)	12	93.3	10.34	88.9	9.1

BUN: Blood urea nitrogen; AST: Aspartate aminotransferase; ALT: Alanine transaminase; ROC: Receiver operating characteristic.

sulphydryl groups, could play a great role in scavenging free radicals[28]. In this research, 33% of the patients were treated with vitamin E, while vitamin C was prescribed for about 30% and NAC for about 48% of the patients. It seems that other researchers in various studies did not choose similar antioxidant medications; Cherukuri *et al*[9] in their study showed that treatment with vitamin E was done in about 18%, while vitamin C was prescribed in 25% and NAC in 50% of their patients. About 15% of the patients in Delirrad's[6] study were treated with NAC. However, in Sabzghabaee[10] and Sandhu *et al*[12]'s studies, all patients were treated with antioxidant medications. In their study on 9 cases, Yasaka *et al*[30] showed that mortality rate in patients who were treated with vitamin E reached 78%, however, in Hong *et al*[31], study on 5 cases they showed that all treated patients survived. Some previous studies have proposed the impact of treatment by pulse corticosteroids and cyclophosphamide in preventing pulmonary fibrosis[31,32]. In this study, 87% of the patients were treated with corticosteroids and 37% with cyclophosphamide. Other researches have shown to use similar antioxidant medications, but they did not use similar immunosuppressive and corticosteroid medications. As Cherukuri *et al*[9] showed in their study, treating with pulse methylprednisolone was carried out in 38% of patients, while treating with cyclophosphamide was carried out in 22% of the cases. In Delirrad's[6] study, 54% were treated with corticosteroids, and 22% of the cases were treated with cyclophosphamide. On the other hand, all patients were treated with corticosteroids in Sabzghabaee[10] and Banday's studies[4].

Our study suggests that consuming more than 22.5 cc of 20% paraquat can lead to poor prognosis in the patients, and this is in accordance with the results of Hosseinian Amiri *et al*[5] and Delirrad *et al*[6], studies. In addition, Buckley *et al*[33] showed that consuming around 10 to 20 cc of this poison could lead to fatal complications.

Thus, results from our study indicates that on average, patients' mortality occurred on the fifth day of hospitalization, which is in accordance with Afzali's[34] study. Also, in 9 cases who had consumed around 35 to 300 cc of the poison, death occurred on the first day of admission. In fact, consuming higher doses of poison can lead to death during the first few hours through acute multi organ failure[1].

Even though hemoperfusion has been introduced as an effective treatment in washing off the poison from plasma in the first 6 h[35], but for the patients in this study hemodialysis was performed, due to lack of hemoperfusion facilities. However, this measure was not performed for any of the patients during the first 6 h of admission. It seems that the main reason for this delay was that they had to wait for receiving the results for viral markers for hemodialysis. Nevertheless, based on Marashi *et al*[36], considering the high mortality rate due to poisoning and the low probability of viral infections, there is no need to study the viral markers, and in such cases hemodialysis should be carried out by the device allocated for patients diagnosed with hepatitis B.

Pulmonary fibrosis is among the known complications in paraquat poisoning which occurs approximately 7 to 14 d after poisoning along with acute respiratory failure[37,38]. This complication transpires due to body's inability to repel the free radicals that leads to the destruction of cell membrane and lipid peroxidation[39]. In this study, among the expired patients, pulmonary fibrosis was the most common radiography finding, which was observed in almost 22% of the patients (more than 55% of the expired patients), which was initially observed on the fourth day. Also, Hsu *et al*[40] showed that pulmonary fibrosis had led to death in about 25% of patients. Our radiography findings in deceased patients were acute respiratory distress syndrome (ARDS), pneumothorax, and pneumomediastinum, which is in accordance with the results of Weng *et al*[41].

Unfortunately, despite various studies, limited variables have been identified for predicting prognosis. Although one of the best prognostic criteria is to determine paraquat serum concentration and to use nomogram[18], but this factor could not be studied due to lack of serum paraquat concentration measurement as a common laboratory test in Iran.

This research showed that the maximum average of serum creatinine levels increased on the third day (up to around 2.5 mg per deciliter), and that the serum creatinine average decreased, subsequently. Serum creatinine level on the third day higher than 1.95 mg per deciliter was accompanied with a poor prognosis in our patients. Ragoucy-Sengler and Pilerire[42] showed that an increase in serum creatinine lower than 0.03 mg during five hours accompanies an acceptable prognosis. On the other hand, Roberts *et al*[43] showed that an increase more

than 0.05 mg during 12 h could lead to poor prognosis. However, according to Levey *et al*[44], creatinine level has insignificant value, even for assessing the kidney damages.

According to other studies, other biomarkers for renal function were not appropriate factors in predicting patients' sequela[23]. This study showed that BUN level higher than 25 on the third day was accompanied with a poor prognosis.

Studying the changes in liver enzymes showed that the maximum average of AST and ALT levels on the third day were 114 and 145, respectively. AST level higher than 24.5 or ALT level higher than 12 on the third day was accompanied with poor prognosis. Considering the fact that these levels are in the normal range for AST and ALT, it seems that they are not appropriate factors for predicting severe poisoning. Furthermore, in their study, Almasi *et al*[45] showed that exposure to paraquat could lead to liver cell damage and increase in AST and ALT levels in rats, which could be treated by prescribing ginger extract. It seems that routine treatment by NAC played a role in improving liver cells' performance and decreased AST and ALT in a number of patients in this study. Since prescribing NAC, as an antioxidant, is an approved treatment for paraquat poisoning, hence it seems that liver biomarkers are not appropriate factors in predicting the patients' sequela.

This research showed that cardiac dysrhythmia is not a common finding in paraquat poisoning, which was in line with the results from Noguchiet *et al*[46]. In contrast, some other herbicides such as glyphosate, glufosinate and chloracetanilide herbicides (*e.g.*, alachlor, metachlor, butachlor, and propanil) appeared to have significant cardiotoxicity[47-51]. Even though other types of herbicides such as; glyphosate, glufosinate and chloracetanilide herbicides are available in Iran, by reviewing the published literature, we could find only one case report of butachlor dermal exposure[52]. It seems that, paraquat as a highly toxic compound is recognized by those who are seeking to commit suicide.

This research showed that a standardized treatment protocol was not used in all paraquat poisoning cases and in some cases, unnecessary or improper measures were carried out for the patients and in contrast or in some cases, a patient was deprived of necessary treatments.

Since there is no charcoal hemoperfusion available in our hospitals, but hemodialysis, which is the alternative choice was not used for extracorporeal elimination in the right time. Therefore, it is necessary to immediately carry out hemodialysis in these patients, along with training physicians and assistants working in teaching hospitals.

It seems that due to lack of paraquat poisoning treatment guidelines, patients are deprived of proper treatment by antioxidant and immunosuppressive medications. Providing treatment guidelines for this type of poisoning could assist in choosing a suitable treatment method. Finally, to better identify this type of poisoning systematic and meta-analysis reviews must be performed.

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COMMENTS

Background

Poisoning with herbicides in developing countries of South, East and Southeast Asia with an agriculture economy is highly common. Paraquat poisoning, is a highly mortal toxicity and rapid management is required to increase patient survival. However, without a standard guideline, there are no agreement on therapeutic strategies conducted in different healthcare facilities. The use of hemoperfusion or hemodialysis during the first hours of admission, followed by administration of immunosuppressive and corticosteroid medications, as well as antioxidants, is currently accepted to be the conventional treatment protocol for these cases. However, in practice, negligence is responsible for low survival rate of patients with paraquat poisoning.

Research frontiers

Reviewing the published literature, it seems that paraquat poisoning has the most prevalence rate in Fars province Iran, amongst different parts of Middle-Eastern countries. The research purpose was to evaluate the accuracy of treatment strategies, conducted to treat this fatal poisoning, as well as to demonstrate prognostic factors regarding long-term survival outcome.

Innovations and breakthroughs

Medical treatment for paraquat poisoning is improving in developing countries. This study represents the largest series of paraquat poisoning cases in the Middle-East ever reported. The current data suggests that therapeutic inaccuracies are common amongst healthcare providers. On the other hand, it was determined that there hasn't been any particular protocol used to treat patients diagnosed with paraquat poisoning. This indicates the necessity to develop a guideline for treating paraquat poisoning in order to provide better healthcare services to these patients. In case there is no access to charcoal hemoperfusion, hemodialysis should be used as an alternative choice in extracorporeal elimination; however, due to delay in reaching the results from viral marker study, hemodialysis was not carried out in most of the patients in the six-hour golden time. The importance of immediate use of extracorporeal removal techniques and omitting the viral markers results, also accurate use of immunosuppressive, corticosteroid and antioxidant medications should be considered as the main treatment protocols.

Applications

Results from this research indicated that the increase in renal biomarkers in the third day could be used in prognosis of the patients; hence, by identifying the patients who are at risk, it could be used as a guideline in intensive treatment measures.

Terminology

Paraquat poisoning is a lethal toxicity in patients who have consumed this herbicide, which is characterized by a rapidly progressive multi-organ failure in severe cases and progressive lung fibrosis in moderate cases. The most important treatment for paraquat poisoning is extracorporeal elimination within the first 6-h of toxicity. By using extracorporeal elimination technics, paraquat ion removal takes place through a machine performing blood circulation outside the body. By using charcoal hemoperfusion, paraquat cleansing will exceed that of hemodialysis. During hemoperfusion, blood is pumped through a cartridge containing activated charcoal.

Peer-review

Kavousi-Gharbi *et al* from Shiraz University of Medical Sciences, Shiraz, Iran investigated cross-sectionally the information about all cases of acute poisoning by the herbicide paraquat (1,1'-dimethyl-4,4' bipyridinium dichloride) admitted

to 3 main teaching hospitals of Shiraz University in a 5-year period (September 2010 to September 2015). A total of 104 patients (66% male) with a mean age 26 ± 11 years were evaluated. The mortality rate was 43%. Despite the necessity of emergency hemodialysis in first 6 h of intoxication, none of the patients had dialysis during this time.

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LETTERS TO THE EDITOR

Comments on eurytrematosis in Brazil and the possibility of human infection

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Abstract

The manuscript "Eurytrematosis: An emerging and neglected disease in South Brazil" discusses some aspects of *Eurytrema sp*. fluke as an animal pathogen and based in some aspects of the parasitism in cattle and the life cycle of *Eurytrema sp*. Authors suggest the possibility of human infection, once there is no research on this subject in Brazil. In human cases reported, the mechanism of infection was not disclosed, so it keeps the discussion opened. Although we focused on animal eurytrematosis, we speculated the possibility of human infection by *Eurytrema sp*. in Brazil, but after all, the only way to determine it, would be a study searching for people infected through coprological or serological tests.

Key words: Veterinary parasitology; Cattle; Pancreas; Eurytrema coelomaticum; Pathology

Core tip: The possibility of human infection by flukes of the genus Eurytrema in Brazil is reviewed. Based on the life cycle of the parasite and the high prevalence of infection in cattle, the possibility is suggested, although only an investigation with coprological or parasitological tests could give some reliable information.

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TO THE EDITOR

The manuscript "Eurytrematosis: An emerging and neglected disease in South Brazil"[1] discusses some aspects of *Eurytrema sp.* fluke as an animal pathogen, regarding it's prevalence, subclinical disease and possible productive losses related to parasitism. Additionally, based in some aspects of the parasitism in cattle and the life cycle of *Eurytrema sp.*, the authors suggest the possibility of human infection. Since this work is

an editorial, the aim was to make comments on an important topic, regarding its current research status and future directions that will promote development of this subject.

We have read with interest the letter to the editor by Pinto *et al*[2]. Although, authors seem to have made an error of interpretation, as they say on the manuscript that eurytremiasis was suggested by Schwertz *et al*[1] to be a neglected and emerging human disease in Brazil. We would like to make clear that our manuscript reviews aspects about bovine's eurytrematosis, and suggests that the disease is neglected and emerging as an important pathogen for cattle in south Brazil, since we basically work with animal diseases. Furthermore, the majority of veterinarians believe the parasite is non pathogenic, information contradicted by us[2,3]. Based on the previously cited arguments, we only suggest the possibility of human subclinical infections, there is no research on this topic in Brazil. Also, at the time of writing the manuscript[1], no molecular identification had been conducted on specimens of *Eurytrema sp.* in Brazil. Based on this information, we speculate the parasite present in Brazil could be *E. pancreaticum*, which could be also present in human beings. Nowadays, our research group has already established by molecular technics that the parasite present in south Brazil is *E. coelomaticum*[3], which is not described as a human pathogen in the literature.

Pinto *et al*[2] criticize the life cycle of *Eurytrema sp.* showed in our editorial, once there is no evidence of infection through the ingestion of metacercariae over the pasture. In fact, it was a mistake to suggest this mechanism of infection without scientific support; although we believe that it could be possible, based on the high prevalence of the parasitism and the questionable probability of accidental ingestion of insects such as *Conocephalus spp.* by 70% of cattle in some regions. The liberation of metacercariae over the pasture by live grasshoppers, in our opinion, could better justify the high prevalence of infection by *Eurytrema sp.* where it occurs, although there is no scientific evidence of this for now. It is inconceivable, to think that 70% of dairy cattle in the area, in 100% of farms, were infected only by the ingestion of these insects. Specially taking into account that when someone walks in the field their agility is noted. Furthermore is quite uncommon to find dead specimens available to be ingested by ruminants. We have found up to 2578 *E. ceolomaticum* flukes in a pancreas of one cattle, and the average was 532[3,4].

According to Headley[5], the fact that *E. pancreaticum* has already been identified in human beings should not be ignored and more epidemiological data must be obtained and analyzed to establish the form of transmission to human beings, thereby discovering the potential of this fluke as a threat to human health. In the case reported by Ishii *et al*[6], it was not possible to determine how the person got infected, but the author presume that she accidentally ingested metacercariae in or from an infected grasshopper.

Pinto *et al*[2] defend that there is no possibility of human infection by *Eurytrema sp.* in Brazil. Still, the species that occurs in Brazil is *Eurytrema coelomaticum*, as we later established[3] and the human cases reported in the literature are due infection by *Eurytrema pancreaticum*[6]. In the editorial[1], we mentioned the possibility of infection but we have not focused on this aspect and we have not discussed this in detail as thoroughly as Pinto *et al*[2]. We have detailed the current research status and future directions of eurytrematosis in cattle. The arguments proposed by them[2] make clear that the possibility of human infection by *Eurytrema sp.* in Brazil is low, but after all, the only way to determine it, would be a study searching for people infected through coprological or serological tests[7].

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